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UNIVERSITY OF ALBERTA

MECHANISM OF ACTION OF PLATELET-ACTIVATING FACTOR ON TWO TYPES OF EPITHELIAL CELLS

BY

PAUL ANTHONY LABRECQUE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

IN

Experimental Medicine

Department of Medicine

Edmonton, Alberta

Fall 1992

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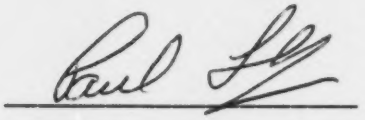

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled MECHANISM OF ACTION OF PLATELET-ACTIVATING FACTOR ON EPITHELIAL CELLS submitted by PAUL ANTHONY LABRECQUE in partial fulfillment of the requirements for the degree of MASTER'S IN SCIENCE in MEDICINE.

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Dedication

I would like to dedicate this thesis to the memory of my loving mother, Hilda Mary Labrecque -- 'Good-bye gentle woman'

I would also like to dedicate my thesis to my family -- father, Alphonse and brothers Mark, Guy and Barry, their families and to my fiancée Michelle -- your love and support give me strength.

Abstract

Platelet-activating factor (PAF) is a potent inflammatory mediator that has been implicated in many pathological conditions. PAF has a number of physiological effects on epithelial cells including increased production of prostaglandins E_2, $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, alteration of short-circuit current and transepithelial resistance in cultured monolayers and increased mucous secretion. The mechanism of PAF action in epithelial cells remains unclear, however, increased intracellular calcium ion concentration ($[Ca^{++}]i$) has been demonstrated in some cell types.

This thesis begins with a comprehensive examination of the literature surrounding PAF, calcium regulation and epithelial cells. The second chapter shows the results from the first study in which I used Madin-Darby canine kidney (MDCK) epithelial cells to examine the effect on $[Ca^{++}]i$ in response to PAF stimulation. The third chapter examines the mechanistic components of the action of PAF on cultured human nasal epithelial explants using a similar methodology to the MDCK study. Finally, in the short concluding chapter I discuss the results and implications of this project.

The first study was to establish a methodology for measuring $[Ca^{++}]i$ in MDCK cells and to examine the changes in $[Ca^{++}]i$ to PAF stimulation. The $[Ca^{++}]i$ in MDCK cells responded in a dose-dependent manner to PAF stimulation. This effect could be reduced by removal of extracellular calcium and could be inhibited by the calcium channel blocker nifedipine and by the PAF receptor blocker WEB 2086.

The second study examined in more detail the mechanism of PAF action in human nasal epithelial cells. The $[Ca^{++}]i$ in these cells also responded in a dose-dependent manner. PAF's effect was diminished when extracellular Ca^{++} was reduced, in the presence of the PAF receptor blockers WEB 2086 and alprazolam and in the presence of n-ethylmaleimide and phorbol 12-myristate 13-acetate -- a G-protein inhibitor and a protein kinase C activator, respectively.

It is hoped that this research has contributed to a better understanding of epithelial cells -- their functions and their role in inflammatory diseases such as asthma.

Acknowledgement

I would like to extend my appreciation to Dr. Peter Pang for the generous use of his Spex 3000 fluoroplex, to Dr. Chris Benishin for the use of her Tracor Fluoroplex III, to Dr. Edward Karpinski and Dan Fackre for their excellent technical advice and cooperation and to Sam Sim whose collaborative efforts on the study of MDCK cells assisted in establishing the experimental protocol. I would also like to thank Robert Francis for his artistic advice and assistance.

In addition, I would like to thank my supervisor Dr. Paul Man for his many hours of advice, encouragement and support. I would like to express my appreciation to the other members of my supervisory committee, Dr. Dale Lien and Dr. Christina Benishin, for their constructive criticism, guidance and insight.

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Chapter 1

Introduction

Introduction

Platelet-activating factor is a lipid-soluble inflammatory mediator that has a diversity of actions; its properties, known and suspected, will be discussed. Calcium is involved in a number of cellular metabolic and regulatory pathways. Cell calcium regulation has been studied in great detail in excitable cells, i.e. cells that carry an action potential, and to a lesser degree in non-excitable cells. In this paper, discussion will focus on extracellular influx, intracellular control and Ca^{++} measurements in non-excitable cells that do not carry an action potential, such as transporting epithelium. Epithelial cells, in particular Madin-Darby canine kidney epithelial cells and primary human airway epithelial cells, will be investigated in terms of their function, morphology and *in vitro* methodology. In each section, particular attention will be paid to airway epithelial cells and aspects that have been influenced by PAF and/or Ca^{++}.

Platelet-activating factor

In 1972, a soluble substance released from IgE stimulated rabbit leukocytes was discovered that had the ability to cause platelet aggregation (Benveniste *et al.*, 1972). The experimental conditions by which this substance was discovered resulted in its being named platelet-activating factor (PAF). It was soon discovered that this name was something of a misnomer -- PAF is one of the most potent and wide ranging phospholipid inflammatory mediators known. PAF is a dynamic chemical with a diversity of actions in a variety of cell and tissue types. Its dynamic chemical nature and potent inflammatory effects make PAF, arguably, one of the most

popular compounds in research today. In order to more fully understand PAF, a review of its many properties will be presented -- its chemical nature, sources (both cellular and tissue), production, function, receptors, signaling mechanism, and its physiological relevance. The physiological relevance section will include current knowledge of PAF's interaction with epithelial cells.

Chemical nature

The basic structure of this phospholipid compound was elucidated in 1980 (figure 1)(Hanahan *et al.*, 1980). PAF consists of a polar headgroup of phosphorylcholine at C3, an ester linked acetyl group at C2 and an ether linked lipid at C1. Drastic alteration of any of these groups or of the stereochemical nature leads to significantly reduced biological activity (Pinckard *et al.*, 1988). The amphipathic nature of PAF gives the compound both hydrophilic and hydrophobic tendencies. The critical micelle concentration (CMC) of PAF is about 10^{-6} M. At concentrations above the CMC it is thought that PAF may exert its actions by disrupting membrane integrity rather than by receptor mediated events (Sawyer and Andersen, 1989). PAF exhibits considerable molecular heterogeneity in its structure. While the most common forms of PAF are 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C16:0-) and 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C18:0-), other alkyl-chain homologues have also been identified -- C15:0-, C17:0-, C18:1-, and C22:2-PAF (Weintraub *et al.*, 1985). Changes in the lipid linkage at position C1 may occur; 1-*O*-acyl-PAF is also produced from some stimulated cells. The acetyl group at C2 has been substituted with propyl and

butyl sidechains; and the polar headgroup at C3 has been substituted with phosphoethanolamine in the place of phosphocholine (Ludwig and Pinckard, 1987). There is still much debate as to the physiological and functional significance of these PAF derivatives. It has been suggested that they serve as modulators to the inflammatory process or as precursors to more biologically active forms of PAF (Pinckard *et al.*, 1988).

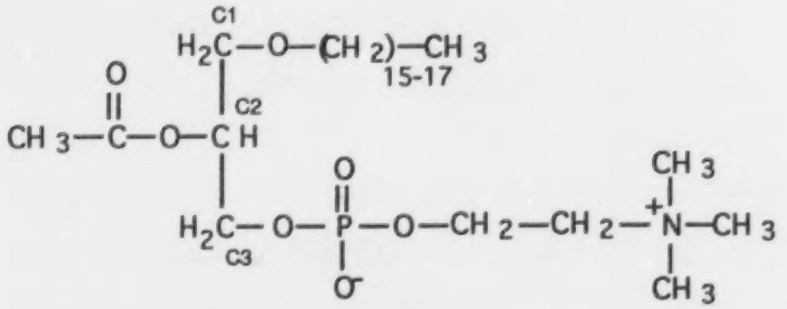

Figure 1. Structure of platelet activating factor (modified from Pinckard *et al.*, 1988)

Sources

PAF is produced by a number of different cell types including monocytes, macrophages, polymorphonuclear leukocytes, platelets (figure 2)(Pinckard *et al.*, 1988), and recently, human airway epithelial cells (Holtzman *et al.*, 1991; Salari and Wong, 1990). A number of tissues including brain, kidney, and lung are also sources of PAF (figure 3)(Pinckard *et al.*, 1988). The list of cells and tissues that produce PAF is still incomplete and will continue to grow in the future. As mentioned previously, a high degree of molecular heterogeneity exists in PAF's structure. In many cases, it has yet to be established which species of PAF different cells and tissues produce and how the different species of PAF affect the cell, tissue

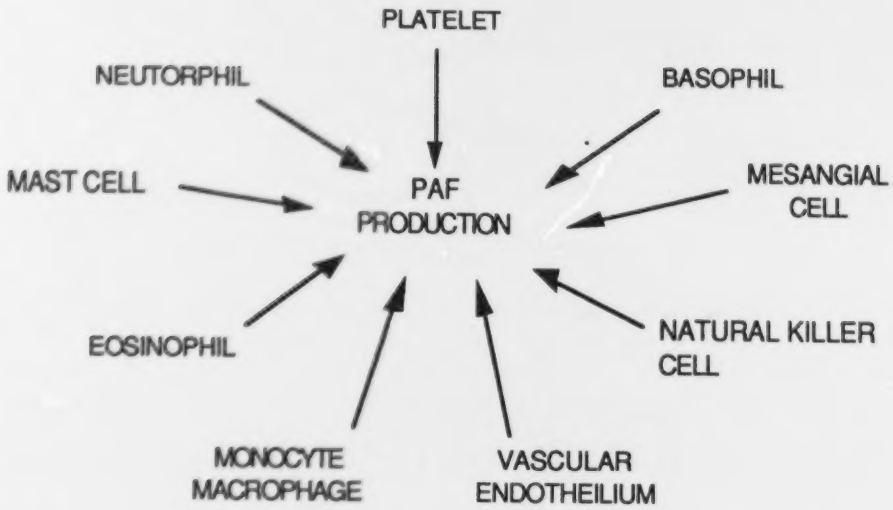

Figure 2. Cellular sources of PAF
(modified from Pinckard *et al.*, 1988)

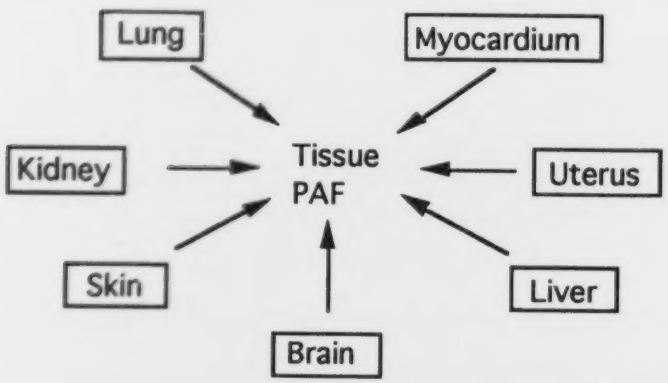

Figure 3. Tissue sources of PAF
(modified from Pinckard *et al.*, 1988)

and organism. Understanding the molecular heterogeneity of PAF and the diversity of cell and tissue types that produce PAF may be important in determining the role each element plays in modulating the inflammatory process.

Production and Secretion

PAF is not stored as a preformed compound within the cell; rather, it is rapidly synthesized by the cell upon stimulation. Intracellular and extracellular calcium levels are the predominant regulatory elements in controlling PAF synthesis. Ca^{++} not only inhibits all enzymes of the *de novo* pathway but also modulates the activities of most enzymes in the PAF biosynthetic pathway (Lee *et al.*, 1986; Lee *et al.*, 1988). For example, phospholipase A2 and acetyltransferase require Ca^{++} for their function (Pinckard *et al.*, 1988).

The two principal pathways for PAF production are: a two step enzymatic deacylation-reacylation reaction and a *de novo* enzymatic pathway (figure 4) (Pinckard *et al.*, 1988). The first pathway, which predominates in most cells, involves initial hydrolysis of long chain fatty acid residues at C2 of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine by activated phospholipase A2, a Ca^{++} dependent enzyme. 1-*O*-alkyl-2-hydroxyl-*sn*-glycero-3-phosphocholine or lyso-PAF is the product formed in the first step. Lyso-PAF, the biologically inactive compound, is then acetylated by acetyltransferase to form the biologically active molecule, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; this is suspected of being the rate limiting step in the process (Pinckard *et al.*, 1988). The biologically active compound is inactivated to lyso-PAF through hydrolysis of the acetyl group by the enzyme acetylhydrolase. The metabolic cycle is completed when lyso-PAF is reacylated with a long-chain fatty acid (usually arachidonic acid) by acyltranferase.

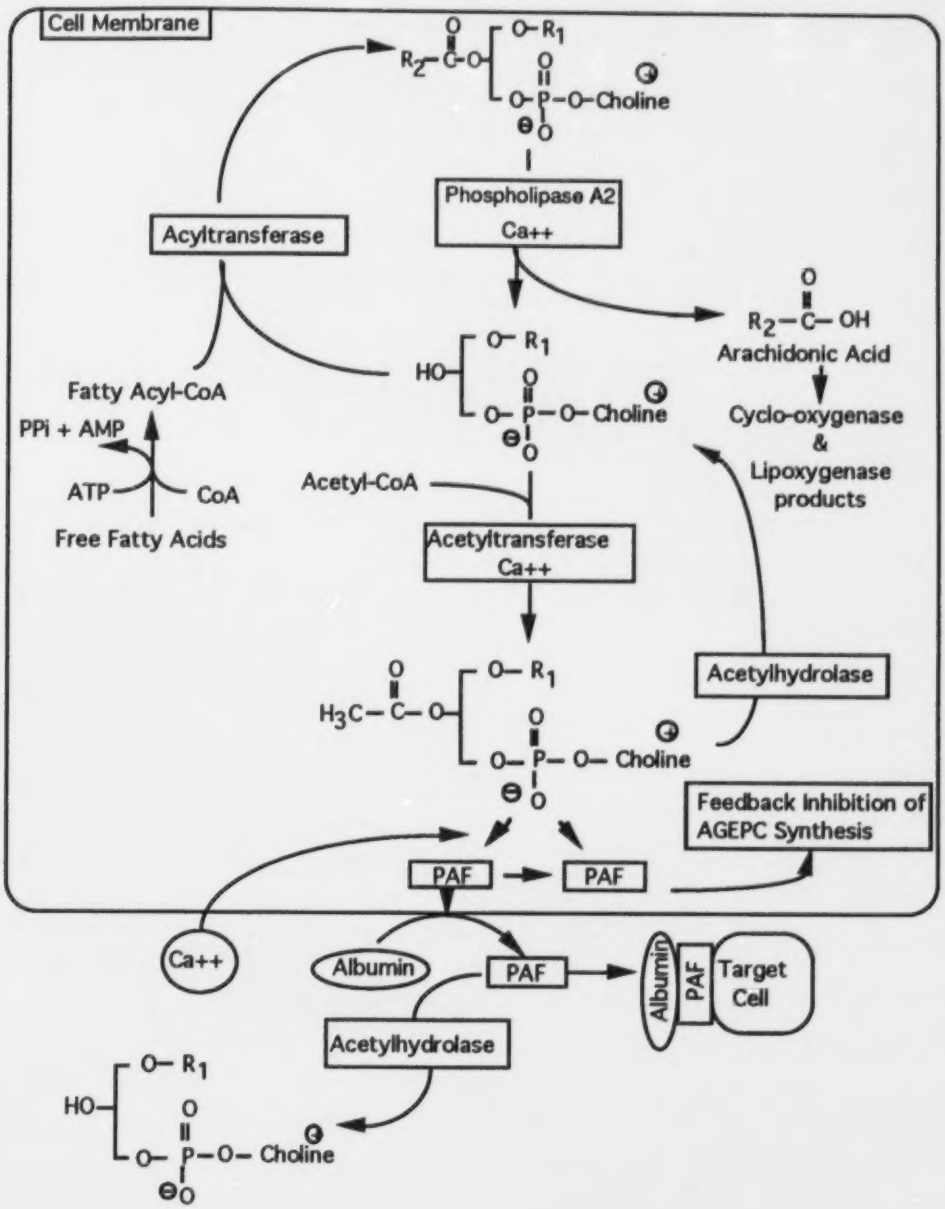

Figure 4. Pathway for PAF Production
(modified from Pinckard *et al.*, 1988)

Cholinephosphotransferase catalyzes the *de novo* pathway which employs CDP-choline and 1-*O*-alkyl-2-acetyl-*sn*-glycerol to form PAF. Stimulation of PAF producing cells results in little acceleration of this pathway. This has led to the speculation that the pathway may play an important role in the production of PAF at basal physiological levels (Lee and Snyder, 1989).

PAF is secreted by all cells that produce it; however, the mechanism of PAF secretion is still undetermined. It appears that two elements regulate the release of PAF -- intracellular and extracellular calcium concentration and extracellular albumin concentration (Ludwig *et al.*, 1985). The extent to which PAF is secreted varies greatly from cell type to cell type. In some cells such as polymorphonuclear neutrophils (PMN's), endothelial cells and monocytes, much of the PAF remains cell-associated regardless of the agonist, agonist concentration, "priming" with lipopolysaccharide or the presence of plasma (Bratton and Hensen, 1989). The reasons for keeping PAF intracellular are unclear; however, some ideas have been proposed. PAF is thought to act upon intracellular receptors and to modulate the metabolic activities of the cell (Bratton and Hensen, 1989). It is also hypothesized that cell-associated PAF is involved in cell-to-cell communication during the inflammatory response (Bratton and Hensen, 1989). In other cell types, such as human PMN's and human airway epithelial cells, PAF release appears to be time dependent and to depend upon the extracellular calcium and albumin concentrations (Pinckard *et al.*, 1988; Holtzman *et al.*, 1991; Salari and Wong, 1990). While the pathways of production have been elucidated, the function of multiple pathways and the control of

these pathways remains unclear. In addition, the exact mechanism and modulation of PAF secretion, and the function of intracellular PAF retention are mysteries that have yet to be resolved.

PAF Receptor

Until recently most studies done on the PAF receptor used radioligand binding assays and classical kinetic analysis (competitive inhibition and Scatchard plot) to determine the existence and characteristics of PAF receptors (Hwang, 1990; Shukla, 1992; Dent *et al.*, 1989). Radioligand binding assays using [^{3}H]-PAF and [^{3}H]-PAF receptor blockers, such as WEB 2086 (Casal-Stenzel *et al.*, 1987) and BN52021, have demonstrated the presence of high and low affinity binding sites and, importantly, receptor heterogeneity in many cell types (Hwang, 1990). The receptor dissociation constant, Kd, (a measure of the affinity of the receptor for the agonist) has shown variation depending on the cell type, species and experimental conditions (Hwang, 1990). Experimental conditions which have been used to modulate binding of [^{3}H]-PAF to its receptor are ionic concentration and GTP (Hwang, 1990). Na^+ and Li^+ ions inhibit [^{3}H]-PAF binding (Na^+ increases the Kd of the receptor). K^+, Cs^+, Rb^+, Mg^{++}, Ca^{++} and Mn^{++} ions all enhance [^{3}H]-PAF binding (Mg^{++} increases both the number and affinity of the receptors). GTP inhibits [^{3}H]-PAF binding to the receptor, presumably through changes in the conformation of the receptor; however, the exact mechanism of this action is unknown (Hwang, 1990). These studies have led to the proposals that ions and GTP modulate the conformation and quantity of the PAF receptor; that multiple conformation sites of the receptor

can exist; that the receptor probably contains a metal ion binding site; and that G-proteins are probably involved in the receptor signaling mechanism.

A major breakthrough in PAF receptor study has recently occurred with the cloning of the PAF receptor gene in guinea pig lung tissue (Honda *et al.*, 1991). The cloned DNA fragment showed a 3020 nucleotide sequence, and the gene product revealed a 342 amino acid sequence (in the longest open reading frame) and a molecular weight of 38,982. Characteristics of the cloned gene product have confirmed elements that have been postulated based upon classic receptor biology techniques. The PAF receptor is a membrane-bound protein that contains seven membrane spanning regions and possesses considerable sequence homology to the superfamily of G-protein receptors (Honda *et al.*, 1991). Several interesting areas in the protein sequence lend insight into the characteristics of the PAF receptor: four serine residues and five threonine residues on the cytoplasmic side may serve as sites of phosphorylation during receptor desensitization; and asparagine residues on the external surface may serve as sites of glycosylation (Honda *et al.*, 1991; Shukla, 1992). The functions of these sites are unclear, and regulatory elements of the receptor need to be fully elucidated. As well, it remains to be seen how the receptor varies between species, cell types and tissue types.

The PAF receptor resides within a superfamily of receptors known as G-protein receptors. In general for these receptors, the message of the agonist is transmitted to the interior of the cell via a guanine-nucleotide (GTP) binding protein -- a G-protein. G-proteins

are heterotrimers composed of 1α, 1β, and 1γ subunit. Isolation of G-proteins from many different cell types has shown that considerable sequence homogeneity exists within the β and γ subunits while amino acid variation occurs mostly in the α subunits (Alberts *et al.*, 1983). The function of G-proteins is to modulate the activity of various cellular enzymes, such as adenylate cyclase and phospholipase C, that are responsible for transmitting the receptor signal to the cellular compartment. Briefly, in unstimulated cells, GDP is bound to the α subunit and all three subunits are linked together. Upon stimulation of the cell by the agonist binding to its receptor, GDP is replaced by GTP. GTP-α dissociates from the β and γ subunits and then associates with the intracellular signalling component (eg. phospholipase C), thereby activating or (in some cases) inactivating the appropriate pathway (Alberts *et al.*, 1983). The signal transduction pathway can also be modulated using compounds that inactivate the G-proteins, such as pertussis toxin or N-ethylmaleimide (Fredholm *et al.*, 1985), or, by using compounds that activate the signal pathway such as cholera toxin. Studies using these pertussis and cholera toxins, GTP inhibition assays and dose dependent hydrolysis of [γ-^{32}P]GTP have provided the majority of evidence demonstrating the involvement of G-proteins in the signal transduction pathway to PAF stimulation (Dent *et al.*, 1989; Hwang, 1990). Confirmation of the G-protein involvement came with the cloning of the PAF receptor gene and categorization of the receptor into a particular family of receptors. The identity of the G-protein for the PAF receptor remains unknown.

Signaling Mechanisms

The PAF receptor activates several signaling mechanisms within the cell including phospholipase C, phospholipase D, phospholipase A2 and protein kinases such as protein kinase C and tyrosine kinase (Shukla, 1992). As previously stated G-proteins play an important role in this activation process. PAF stimulates the turnover of phosphatidylinositol in many cell types (Pinckard *et al.*, 1988). Phospholipase C (PLC) is a membrane-bound enzyme that is activated when the dissociated GTP-α G-protein binds to the inactive enzyme. Activation of PLC causes the degradation of membrane bound phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Berridge and Irvine, 1989). In general, within several seconds after stimulation the IP3 and DAG concentrations increase, peaking at about 60 seconds post-stimulation. DAG remains associated with the plasma membrane where it effects the translocation of PKC to the plasma membrane and helps to maintain the PKC in the membrane bound state (Rasmussen, 1989). IP3 diffuses into the cytoplasm of the cell where it mobilizes endogenous, membranous stores of intracellular calcium, such as the endoplasmic reticulum and calciosomes, leading to an increase in the free Ca^{++} ion concentration, $[Ca^{++}]i$ (Berridge and Irvine, 1989). The increase in $[Ca^{++}]i$ from intracellular stores accounts for about one fourth of the total increase seen in MDCK epithelial cells (De Smedt *et al.*, 1988). This indicates that for a sustained PAF response there is an influx of Ca^{++} from the external environment. The mechanism for this influx is unknown but is thought to be due to a receptor-activated calcium channel. IP3 is

metabolized through a phosphorylation-dephosphorylation pathway to other compounds such as IP4 and IP5 (Berridge and Irvine, 1989). These other phosphoinositol metabolites are suspected of having modulatory roles in controlling the calcium cascade within the cell. Exact roles for these compounds are the focus of intensive research at the present but remain undetermined at this time.

PAF stimulation in many cell types increases the production of arachidonic acid metabolites such as prostaglandins and thromboxanes. This occurs via activation of phospholipase A2, (PLA2)(Kawaguchi and Yasuda, 1986; O'Flaherty and Wylke, 1989). The increased production of these metabolites can be blocked using PAF receptor antagonists, PLA2 inhibitors, and tyrosine kinase inhibitors (Shukla, 1992). This suggests that the PAF receptor transduction pathways activate PLA2 and that tyrosine kinase may play a modulating role in this activation. At the present time, however, it is unclear what these results mean. The function, control and interaction of the many pathways involved in PAF stimulation need to be determined.

Phospholipase D has only recently been implicated in the PAF response mechanism. Its activation was marked by the production of phosphatidylethanol in the presence of ethanol (Shukla and Halenda, 1991). The relative contribution of PLD in the cellular response to the PAF response in stimulated cells remains to be established.

In PAF responsive cells protein phosphorylation has led to the involvement of protein kinase C (PKC) and possibly tyrosine kinase in the PAF response (Dhar *et al.*, 1990). The increase in the $[Ca^{++}]i$ and DAG from PLC activation causes PKC to migrate from the cytoplasm to

the plasma membrane. PKC is responsible for the phosphorylation of many proteins (Block *et al.*, 1989). It is thought that PKC phosphorylates a calcium-ion pump, thus increasing the efficiency of Ca^{++} cycling between the cell and the extracellular space. This cycling is critical for maintaining a sustained cellular response to stimulation; as well as preventing the $[Ca^{++}]i$ from becoming too high and potentially cytotoxic (Rasmussen, 1989). Recently, tyrosine phosphorylated proteins have been identified in some cells, possibly indicating the action of tyrosine kinase in these PAF activated cells (Dhar *et al.*, 1990). The exact roles for these two protein phosphorylation enzymes remain to be identified.

Physiological relevance

PAF is the most potent phospholipid agonist known to date. It is involved in many pathophysiological events such as inflammation, asthma, bronchial hyperresponsiveness and cardiovascular disease (figure 5)(Pinckard *et al.*, 1988). Asthma is a disease that is receiving a great deal of attention due to the increasing morbidity rate and the pathogenic uncertainty surrounding it (Costello, 1991). The complex inflammatory process associated with asthma is under intense investigation and much of the attention is focusing on PAF (Barnes *et al.*, 1989). Elements of asthma that are associated with the inflammatory process are contraction of the airway smooth muscle, microvascular leakage, mucous hypersecretion, edema, plasma extravasation and epithelial damage (Barnes, 1991). Inflammatory mediators like histamine, bradykinin and the prostaglandins, have been implicated in the pathogenesis of asthma but PAF is the only

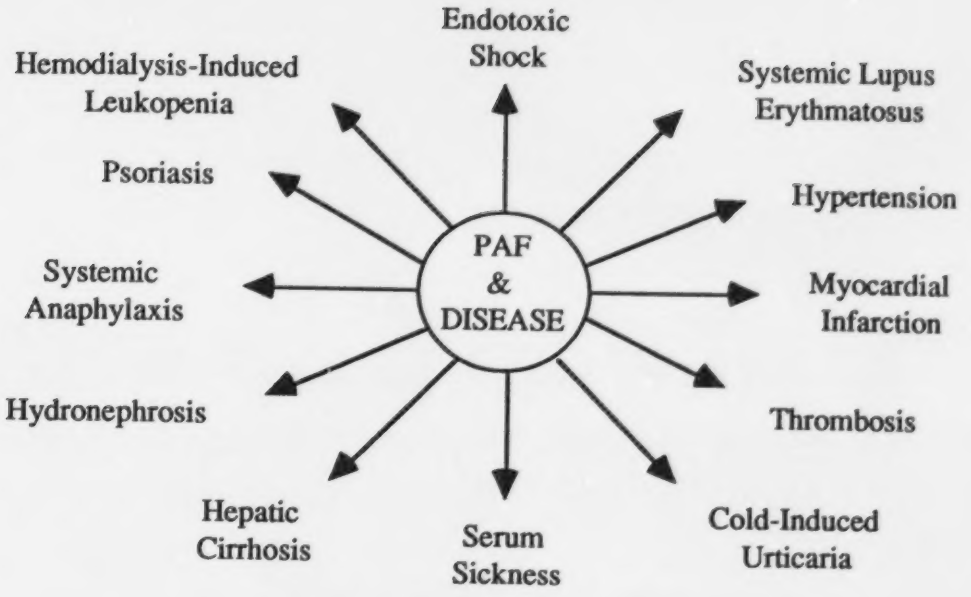

Figure 5. Possible PAF Association to Disease (modified from Pinckard *et al.*, 1988)

mediator which has been shown to cause sustained bronchial hyperresponsiveness (Cuss *et al.*, 1986; Rubin *et al.*, 1987). There are many theories, implications, temporal relationships and associations surrounding the physiological actions of PAF; however, a number of factors limit the mechanistic understanding of the PAF actions *in vivo*. The rapid metabolism of PAF by many cell types and lack of adequate assay techniques make detecting PAF *in vitro* (such as using bronchial alveolar lavage) very difficult (Bratton and Hensen, 1989). Following the physiological action of PAF is impossible, at the present, due to the large number of cells involved, possible stimulation of neural signaling pathways, the activation of multiple cellular signaling pathways and the concomitant production of other active compounds (such as leukotrienes, prostaglandins and thromboxanes) in the PAF response (Barnes *et al.*, 1989). These

factors make data interpretation of *in vivo* experiments questionable and determination of PAF's direct and indirect physiological effects very difficult.

The effects of PAF on epithelial cells have been investigated in a number of *in vitro* experimental models. Renal epithelial cells (MDCK) respond to PAF by releasing incorporated [^{3}H]-arachidonic acid -- this shows the activation of phospholipase A2; by stimulating prostaglandin (PG) E_2 synthesis -- this demonstrates increased arachidonic acid metabolism by the cyclooxygenase pathway; and by increasing the amount of diacylglycerol in the membrane -- this indicates increased activity of phospholipase C (Kawaguchi and Yasuda, 1986). In airway epithelial cells, PAF elicits a number of metabolic changes. Guinea pig, canine and human tracheal epithelial cells increase the release of prostaglandins E_2, $PGF_{2\alpha}$, and 6-keto $PGF_{1\alpha}$ and thromboxane B_2 upon PAF stimulation (Widdicombe *et al.*, 1989; Churchill *et al.*, 1991). In addition, PAF stimulates the short-circuit across dog tracheal epithelium (Widdicombe *et al.*, 1989; Tamaoki *et al.*, 1991); but does not change the short-circuit current across canine bronchial epithelium (Tamaoki *et al.*, 1991). Changes in short circuit current are directly related to Cl^- ion flux across epithelial cells (Welsh, 1987). Feline tracheal epithelial cells increase mucin release when stimulated with PAF (Rieves *et al.*, 1992). PAF is produced by human and ovine tracheal epithelial cells (Holtzman *et al.*, 1991) and by a human type II alveolar epithelial cell line (Salari and Wong, 1990) but not by guinea pig tracheal epithelial cells (Churchill *et al.*, 1991). Some effects appear to be common to all cells tested -- prostaglandin synthesis and short-circuit current

stimulation. However, species differences in arachidonic acid metabolism have been documented (Eling *et al.*, 1988); and it appears that there are species differences in PAF production (Churchill *et al.*, 1991; Holtzman *et al.*, 1991). In addition, PAF's effects may be limited to certain portions of the airways (Tamaoki *et al.*, 1991). More investigation is needed to understand these differences; to elucidate the mechanism of PAF's action on epithelial cells; and to understand the consequences of PAF stimulation of epithelial cell pathways.

Calcium

Calcium ions (Ca^{++}) regulate many cellular functions such as smooth muscle contraction (Karaki and Weiss, 1988), mast cell mediator release (Foreman *et al.*, 1977), mucous secretion (Richardson *et al.*, 1985), and metabolic pathways. Changes in the intracellular calcium ion concentration, $[Ca^{++}]i$, are an integral part of coupling agonist stimulation to cellular response. These changes in the $[Ca^{++}]i$ come about through an influx of Ca^{++} from the extracellular environment and through mobilization of intracellular stores. Fluorescent dyes such as fura-2 are used to quantitate the $[Ca^{++}]i$ (Grynkiewicz *et al.*, 1985). This dye (and others like it) have been used recently to greatly enhance the understanding of cellular calcium response to agonist stimulation under experimental conditions. Since many cellular activities are regulated by the $[Ca^{++}]i$, this understanding sheds new insight into the roles that the cells play in more complex physiological responses. Discussion will be limited to the regulation of calcium in non-stimulatory cells, i.e. cells

that do not fire action potentials; measurement of $[Ca^{++}]i$ will be confined to the use of fura-2.

Calcium influx

Outside of the neuromuscular system there are many transporting epithelial cells that do not elicit action potentials, and actually hyperpolarize rather that depolarize when stimulated with hormones (Pfeilschifter *et al.*, 1991). It is important though, to realize that these cells may contain ion channels that are modulated or regulated in some fashion by the membrane potential (Petersen, 1990). The molecular mechanism of Ca^{++} entry in these cells is not known; but some facts have been established. Cells maintain an $[Ca^{++}]i$ about 100 nM while the extracellular $[Ca^{++}]$ is approximately 1 mM -- this represents about a 10,000 fold gradient of calcium between the interior and exterior of the cell. The cellular membrane is impermeable to calcium; therefore, Ca^{++} must be brought into the cell through specific channels upon cell stimulation (Petersen, 1990). These channels are of 2 types, receptor-operated Ca^{++} channels and second-messenger-operated Ca^{++} channels, and may come in 3 forms Ca^{++}-selective voltage-gated channels, Ca^{++}-selective voltage-insensitive channels and non-selective voltage-insensitive channels (Petersen, 1990). The search for receptor-operated Ca^{++} channels has not been successful probably because the current is very small (Llano *et al.*, 1987). It has been suggested that non-selective cation channels (Petersen and Maruyama, 1983; Von Tscharner *et al.*, 1986) or Ca^{++}-selective voltage-insensitive channels (Zschauer *et al.*, 1988) may play a role in receptor-operated Ca^{++} influx; however, no

experimental evidence exists to support these suggestions (Petersen, 1990). Recently, it has been shown that voltage-gated (L-type) Ca^{++} channels in adrenocorticotropic cells and in insulin secreting cells can be activated by hormone receptors through a G-protein mechanism (Hescheler *et al.*, 1988; Velasco *et al.*, 1988). In addition, there is strong, but indirect, evidence for second-messenger operated Ca^{++} channels in electrically non-excitable cells (Berridge and Irvine, 1989). The inositol phosphate (IP) family of compounds, in particular IP3 and IP4, are suspected of initiating Ca^{++} influx through membrane channels. The major problem at this time is showing a direct effect by the IPs and defining which of the IPs are involved and exactly what role they play in Ca^{++} influx (Petersen, 1990). There are several mechanisms for regulating Ca^{++} entry into cells following receptor activation. In the stimulated cells there are probably several systems operating simultaneously. At this point more research is required to clarify the mechanism of Ca^{++} entry into non-excitable cells.

Intracellular Ca^{++} control

An extensive network within the cells is responsible for adjusting the $[Ca^{++}]i$. $[Ca^{++}]i$ is regulated by compartmentalization of the Ca^{++} across cellular membranes and by using cytoplasmic buffers. These two components allow for rapid and selective access to Ca^{++} on demand and maintain the large concentration gradient across the plasma membrane (Muallem, 1990). Ca^{++} enters across the plasma membrane, as described above, through a number of possible channels; efflux of calcium out of the cell occurs through the plasma-

membrane Ca^{++} pump and the Na^{+}/Ca^{++}-coupled exchange system. The pump accounts for most of the Ca^{++} efflux from the cytoplasm of resting non-excitable cells, but the relative contribution of the two systems is difficult to determine since no selective inhibitors are available (Muallem, 1990). The pump is located in the plasma membrane and in the membrane of intracellular organelles. Hormone stimulation activates the Ca^{++} pump 5 to 7 fold (Korchak *et al.*, 1984) and reaches maximal activity in 1.5 to 2 minutes post-stimulation (Muallem *et al.*, 1988b). This activation appears to come from protein kinase C phosphorylation (Neyses *et al.*, 1985) although calmodulin is also suspected of activating the pump in a hysteresis-like manner (Foder and Scharff, 1981). Several isoforms of the plasma-membrane Ca^{++} pump have been identified using cDNA clones and a calmodulin-binding sequence is located near the C-terminal region (Verma *et al.*, 1988; Shull and Greeb, 1988). The significance of several Ca^{++}-pump isoforms is unknown and additional research is necessary to elucidate the exact mechanisms controlling the Ca^{++} pump.

Intracellular Ca^{++} stores come in two forms, cytoplasmic buffers and intracellular membranous organelles. The main function of intracellular stores is to prevent large fluctuations in the $[Ca^{++}]i$. The Ca^{++} buffers within the cell cytoplasm include: phosphate-containing molecules such as nucleoside phosphate ($Kd = 10^{-5}$ M); high affinity Ca^{++}-binding proteins (Cruetz *et al.*, 1983; Moore and Dedman, 1982; Shadle *et al.*, 1985; Sudhof *et al.*, 1984; Sugden *et al.*, 1979); and acidic phospholipids that have significant Ca^{++}-binding capacity (Dowson, 1965). Intracellular organelles are the endoplasmic reticulum (ER), the mitochondria (Berridge and Irvine, 1989) and the

recently described calciosome (Volpe *et al.*, 1988). These organelles are the most important components of $[Ca^{++}]i$ regulation because of their capacity to actively accumulate and store Ca^{++}; therefore, adequate functional control of these organelles is necessary to prevent large fluctuations in the $[Ca^{++}]i$. The ER stores Ca^{++} in distinct fractions (Streb *et al.*, 1983), the mitochondria stores Ca^{++} as a precipitate of $CaPO_4$ and the calciosome contains an unidentified Ca^{++}-binding protein that is immunologically similar to calsequestrin (a Ca^{++}-binding protein in muscle sarcoplasmic reticulum)(Volpe *et al.*, 1988). Activation of phospholipase C (PLC) results in the generation of inositol 1,4,5-trisphosphate (IP3) and other IP compounds that are known to release Ca^{++} from intracellular stores (Berridge and Irvine, 1989). In the ER, for example, IP3 binds to a receptor on the ER membrane which increases the membrane Ca^{++} permeability (by at least three orders of magnitude)(Muallem *et al.*, 1988a). The Ca^{++} is released through a K^{+}-electrochemical pathway (Muallem *et al.*, 1985). High Ca^{++} concentrations appear to uncouple IP3 from its receptor; thus inhibiting further IP3 mediated release from intracellular stores (Cheuh and Gill, 1986; Jean and Klee, 1986). In addition, high Ca^{++} stimulates a kinase that phosphorylates IP3 to IP4 presumably reducing the amount of IP3 available for internal receptor stimulation. Inositol-1,3,4,5 tetrakisphosphate (IP4) is postulated to have an important role in opening Ca^{++} channels on the plasma membrane to allow influx of Ca^{++} from the external environment (Berridge and Irvine, 1989). GTP also releases intracellular Ca^{++} stores by an unknown mechanism that is different from the IP3 mediated release (Veda *et al.*, 1986). Upon termination

of cell activation, the Ca^{++} stores must be reloaded. This process is dependent on continued extracellular Ca^{++} influx (Merrit, 1987). The exact mechanism of the Ca^{++} reloading pathway is undetermined at this time. Regulation of $[Ca^{++}]i$ is one of the most crucial processes for any cell. In non-excitable cells such as transporting epithelium, this process requires much research to elucidate the mechanisms of Ca^{++} influx, efflux and release of intracellular stores.

Calcium measurement

The invention of improved calcium-chelating fluorescent dyes, in 1985, has made it possible to reliably and accurately measure the intracellular calcium ion concentration (Tsien, 1989). One of the most common fluorescent indicators is fura-2 -- a derivative of EGTA (Grynkiewicz *et al.*, 1985). It has many unique qualities that make it particularly useful for measuring $[Ca^{++}]i$ (Cobbold and Rink, 1987): the absorption coefficient and quantum yield make it 30 times brighter than its predecessor quin-2; the excitation is red-shifted out of the ultra-violet range enough that normal glass microscope optics can be used; there is a marked excitation shift from 380 nm to 340 nm upon Ca^{++} binding to fura-2; the dissociation constant, Kd, is 224 nM (higher that Quin-2) -- this allows for more accuracy in the Ca^{++} readings; fura-2 has a better Ca^{++}/Mg^{++} selectivity that virtually eliminates Mg^{++} interference; fura-2 photobleaches more slowly that does quin-2. Drawbacks of fura-2 have been identified (Malgaroli *et al.*, 1987): significant accumulation of fura-2 acetoxymethyl ester into intracellular organelles; incomplete hydrolysis of the ester moieties; and significant dye leakage from certain cell types.

Solutions to these problems are available (Malgaroli *et al.*, 1987). Dual-wavelength excitation/ emission and the ratio analysis are used to quantitate $[Ca^{++}]i$ from fura-2 intensity counts. Briefly, the cells are alternately stimulated with 340 nm and 380 nm wavelength light. Intensity counts at 340 nm are divided by the intensity counts at 380 nm to give the ratio (R). The ratio is related to the calcium concentration by the formula (Grynkiewicz *et al.*, 1985):

$$[Ca^{++}]i = Kd * \left(\frac{R - Rmin}{Rmax - R}\right) * \left(\frac{Sf2}{Sb2}\right)$$

Rmax is the ratio when fura-2 is saturated with calcium and Rmin is the ratio when fura-2 is free of calcium. Sb2 and Sf2 are the 380 nm intensity counts at Rmax and Rmin respectively. This technique is simple, effective and the technology is readily accessible. Fura-2 has been used in a wide variety of cells including airway epithelial cells to assess $[Ca^{++}]i$ changes in cellular response to agonist stimulation. Because Ca^{++} affects many cellular events, this insight has helped to determine some cellular functions under normal and pathological conditions (Murphy *et al.*, 1988).

The role of calcium in the metabolic pathways of epithelial cells has been investigated in a number of physiological systems. In the MDCK epithelial cells, various agonists have been used to investigate calcium regulation. Calcium modulates the production of tight junctions between epithelial cells (see discussion on epithelial cell function below). Hormones, such as adrenaline (Pfeilschifter *et al.*, 1991, Lang *et al.*, 1988), bradykinin (Lang *et al.*, 1988) and calcium

ionophore (Lang *et al.*, 1988; Winter *et al.*, 1991) have been used to elucidate the mechanism of calcium regulation and hormone action in these cells. These studies have identified cellular Ca^{++} pumps that help to regulate intracellular calcium flux (De Smedt *et al.*, 1988); Ca^{++}-activated K^+ channels involved in Cl^- ion flux (Welsh, 1987); and the role that cytosolic calcium may play during pathological events such as ischemic acute renal failure (McCoy *et al.*, 1988). Intracellular calcium also influences airway epithelial cell function in many ways. Secretion of surfactant by rat alveolar type II epithelial cells is regulated by the $[Ca^{++}]i$ (Dobbs *et al.*, 1986). Electrolyte transport across airway epithelium is regulated by Ca^{++} (Hartmann *et al.*, 1992; Welsh, 1987); and the effects of various hormones and Cl^- secretagogues on $[Ca^{++}]i$ have been investigated in human airway epithelial cells (Harris *et al.*, 1991; Liedtke, 1992; Paradiso *et al.*, 1991). In addition, extracellular Ca^{++} regulates growth and differentiation of monkey tracheal epithelial cells in culture (Martin *et al.*, 1991). Intracellular $[Ca^{++}]$ has been measured using fura-2 in normal and diseased (cystic fibrosis) epithelial cells (Murphy *et al.*, 1988); identifying changes in $[Ca^{++}]i$ under these conditions will provide insight into airway epithelial cell function and biology.

Epithelial cells

Epithelial cells form the lining of many organs within the body; isolation and characterization of the cellular properties gives insight into the role that the cells play in complex physiological responses. Cultured cells come in two forms -- a cell line or primary cells (Davis *et al.*, 1980). Madin-Darby Canine Kidney (MDCK) is an epithelial cell

line. A cell line grows from one immortal, often cancerous, cell that has similar qualities to the native cell type. Primary cells are cultured directly from native tissue. These cells are (at least initially) identical to the cells found in the native tissue. Primary cells often dedifferentiate after a short time in culture and have a limited life span in culture, i.e. they are mortal (Davis *et al.*, 1980). The airway epithelium discussion below refers almost exclusively to native tissue or primary cells.

Madin Darby Canine Kidney Cells

Madin Darby Canine Kidney (MDCK) is an epithelial cell line that was derived from a canine kidney carcinoma in 1958 (Madin and Darby, 1958). MDCK cells resemble those cells found in the distal nephron; and demonstrate responses similar to native cells. These cells have been used for a variety of studies from transepithelial transport (Rindler *et al.*, 1979) to intracellular signaling mechanisms (Lang *et al.*, 1988; De Smedt *et al.*, 1988). Hormonal effects and calcium transport systems have also been examined in these cells (Pfeilschifter *et al.*, 1991). MDCK cells respond to agonist stimulation in a manner typical of non-excitable cells. Extracellular Ca^{++} influx is through as yet unidentified Ca^{++} channels; and intracellular calcium is mobilized via the classic receptor-mediated IP3 mechanism (Lang *et al.*, 1988). IP3 accounts for about 25% of the accumulated calcium during hormone stimulation (De Smedt *et al.*, 1988). Two ATP-driven Ca^{++}-pumps found in MDCK cell membrane preparations are thought to play roles in the control of Ca^{++} movement (De Smedt *et al.*, 1988). Cell lines, such as MDCK, serve as useful models to investigate many

aspects of cellular activation and function. The advantages of a cell line are its cellular homogeneity and bountiful supply. However, its cancerous origin raises questions about the limits of the cells similarity to native tissue.

Airway epithelial cells

The airway epithelium is made up of different types of cells that line the respiratory system and fulfills a variety of functions. These functions and the morphology and cells of the respiratory epithelial lining will be outlined. A discussion highlighting the recent developments in the tissue culture of epithelial cells will follow as these *in vitro* techniques have allowed for great progress in understanding the role that these cells play under normal and pathological situations.

Function

Airway Epithelium consists of a heterogeneous population of cells, and covers the respiratory system to form a continuous lining for the conducting airways. The lining performs many functions (Rennard *et al.*, 1991). First, tight junctions that exist between the apical surfaces of the epithelial cells form an impermeable barrier to undesirable elements from the external environment. In addition, tight junctions allow the cell to contain some cellular functions to the apical or basolateral side of the cell; thus polarizing cellular functions like Na^+ absorption (basolateral) and Cl^- secretion (apical). Second, secretions from the epithelial cells form a biphasic fluid on the lumenal side that consists of an aqueous "sol" phase next to the cell

surface that contains proteins, lipids and ions and of an outer gel phase containing mainly mucus. Third, debris is trapped in the mucus and is constantly being propelled upward and out of the lungs by ciliated cells that predominate in the normal airway. The fourth function of the epithelium is to repair itself in the event of injury. Finally, the epithelium produces metabolic products that may modulate the function of other airway components such as smooth muscle, inflammatory cells and blood vessels (Rennard *et al.*, 1991). Alteration of some or all of these epithelial functions contributes to disease manifestation in asthma and cystic fibrosis.

Airway Morphology

In the nasal passages, trachea and bronchus, the epithelium has a pseudostratified columnar appearance (figure 6). At least 12 different types of epithelial cells have been described on the surface epithelium in these areas; they fall into three broad categories -- basal cells, ciliated cells and secretory cells (Breeze and Wheedon, 1977). Basal cells and intermediate cells are small cuboidal cells that are attached to the basement membrane but do not reach the surface of the epithelium. They are thought to be precursor cells in the epithelium. The ciliated cells predominate; and are tall columnar cells with beating cilia responsible for clearing mucous proximally (out of the lung). Ciliated cells are terminally differentiated and do not divide. Secretory cells are also columnar cells which contribute to the production of mucous (the main mucous production comes from submucosal glands under the surface epithelium)(Weibel, 1988). Secretory cells occupy a small percentage of the cells on the surface

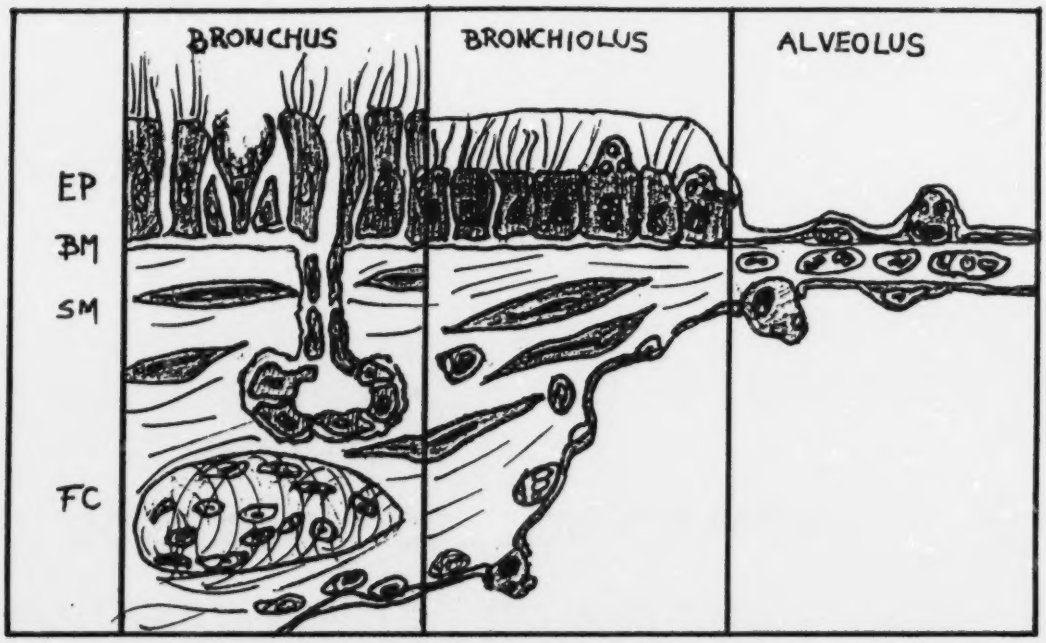

Figure 6. Schematic of Airway Morphology
(modified Weibel, 1988)

epithelium; and may also serve as stem cells. Several types of secretory cells exist including Clara cells, serous cells, mucous cells, and goblet cells. The pseudostratified appearance of the epithelium comes from the different heights of the cells in the epithelial lining. In the bronchioles, basal cells disappear, the height of the epithelium decreases (in proportion with the airway diameter) and ciliated and Clara cells predominate (Rennard *et al.*, 1991). The alveoli contain 2 cell types the non-proliferative type-1 cell and the proliferative type-2 cell. Type-1 cells are terminally differentiated, occupy about 97% of the alveolar surface and are responsible for gas exchange across the epithelium to the blood stream (Weibel, 1988). Type-2 cells are responsible for the production of surfactant and is the stem cell for type-1 cells. The anatomical morphology of the airway

epithelium has been well characterized (Breeze and Wheedon, 1977; Rennard *et al.*, 1991; Weibel, 1988); however, histological classification of the heterogeneous population presents some limitations that make identifying the different cell types difficult: subtle distinguishing factors between the cells may lead to incorrect identification; special staining techniques or electron microscopy may be required; gradual blending of features from one cell type to another (Rennard *et al.*, 1991). Monoclonal antibodies to specific cell markers would assist in definitive identification (Rennard *et al.*, 1991).

Methodology

Development of techniques for maintaining airway epithelial cells *in vitro* have been instrumental in successfully studying airway epithelial cell biology. While not without its drawbacks (see below), tissue culture of these cells has spawned studies on the roles of airway epithelial cells in complex physiological processes (Hartmann *et al.*, 1992; McCoy *et al.*, 1988). These studies have led to insight into epithelial cell function under normal and pathological conditions (Murphy *et al.*, 1988; Welsh, 1987). Advances in culture techniques have permitted partial definition of growth factors and hormones that are required to maintain the differentiated cells in culture (Yankaskas *et al.*, 1985; Wu *et al.*, 1985; Adler *et al.*, 1987; Jetton, 1991). More research is necessary to completely define which factors maintain cellular differentiation *in vitro* (Alpert and Walenga, 1991). At present, this knowledge void limits the progress in some areas of epithelial cell research (see below). Cultured epithelial cells have

been used to successfully study the various aspects of epithelial function: the barrier function (Schneeberger, 1991); epithelial ion transport (Welsh, 1987); mucous secretion (Rieves *et al.*, 1992); cilia activity (Tamaoki *et al.*, 1988); hormone interaction (Harris *et al.*, 1991) and the regulatory effects of metabolic products on other airway components (Yu *et al.*, 1992). Information from these areas has greatly added to the body of knowledge of epithelial cells; however, complete understanding of the role of the epithelial cells and of the epithelium *in vivo* is contingent upon the resolution of certain problems.

There are still several problems with epithelial tissue culture that need to be resolved. Further definition of the hormones and growth factors involved in cellular differentiation is necessary in order to maintain the cells in a highly differentiated state for long periods of time; and to claim that the cultured cells are representative of the native epithelium (Alpert and Walenga, 1991; Jetton, 1991; Martin *et al.*, 1991). There exists a plethora of culture techniques and these are not used consistently throughout the field. In addition, species differences articulate the dilemma (Rennard *et al.*, 1991). The heterogeneity of the cell population creates problems defining the roles of the specific cell types under normal and pathological conditions (Rennard *et al.*, 1991). Gradient centrifugation (Takizawa *et al.*, 1989), and flow cytometry (Aitken *et al.*, 1991) techniques have been marginally successful at isolating cell types; however, they do not result in pure cell suspensions and, therefore, influence from contaminating cells can not be ruled out (Rennard *et al.*, 1991). These problems have led to difficulties in integrating and

interpreting the data gathered. Lack of adequate techniques has slowed progress in attempts to isolate the stem cell(s) for the diverse epithelium; to define cell-to-cell communication; and to establish the metabolic and regulatory roles for the various cell types under normal and pathological circumstances. Developing techniques that allow homogeneous populations of airway epithelial cells to be kept in culture for long periods of time is necessary in order to more adequately describe their biology (Rennard *et al.*, 1991).

Conclusion

The literature presented here has provided background and current knowledge on three major topics -- platelet activating factor, cell calcium regulation and epithelial cells. Platelet activating factor has been the topic of much interest in recent years. This interest has provided much information on its structure, function and properties (*in vivo* and *in vitro*). However, as discussed in this paper there are many characteristics of this chemical still to be uncovered. Cellular regulation of intracellular calcium is, arguably, one of the most critical control mechanisms to the cell. Yet, very little is known on this issue in non-excitable epithelial cells. Broad categories of calcium channels have been described -- only tiny bits of specific information are available on mechanisms of calcium influx and control in this type cell. Epithelial cells are wide ranging cells found in most organ systems of the body; yet, in many systems, like the respiratory system, the roles and regulatory mechanisms of the epithelial cell in normal and pathological conditions remain unknown. What mechanisms are at work? Are the mechanisms the same in all species? all cell types? all organ system? To coin a ubiquitous sentiment in the literature... more research must be done to answer these questions.

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Chapter 2

Effect of PAF on $[Ca^{++}]i$ in MDCK Epithelial cells

Introduction

Identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, platelet-activating factor (PAF) is one of the most potent inflammatory mediators known. PAF elicits a number of effects in epithelial cells. In MDCK epithelial cells, which resemble those of the distal nephron (Madin and Darby, 1958), PAF increases the production of prostaglandin E_2 and increases the activity of protein kinase C and phospholipase C (Kawaguchi and Yasuda, 1986). In airway epithelial cells, PAF alters the short-circuit current and transepithelial resistance in cultured monolayers (Tamaoki *et al.*, 1991; Widdicombe *et al.*, 1989), increases production of PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 (Churchill *et al.*, 1991; Widdicombe *et al.*, 1989), and causes airway mucous secretion (Rieves *et al.*, 1992).

While the physiological effects of PAF are known in many systems, the cellular mechanisms of action of PAF in epithelial cells remains to be established. Specific PAF receptors and receptor subtypes exist in many cell types and may also play a role in the epithelial response to PAF (Hwang, 1990). Evidence for this involves demonstrating saturable binding of PAF to cells and membranes, specific desensitization (tachyphylaxis) of the cell response and inhibition of the response by specific receptor antagonists (Henson, 1989; Hwang, 1990). It has been shown in other cell types that PAF receptors activate, via G-proteins, the phosphatidylinositol pathway, with a concomitant increase in $[Ca^{++}]i$ (Pinckard *et al.*, 1988; Henson, 1989; Hwang, 1990). These mechanisms could also be important in epithelial cells.

Calcium mobilization may be an important element in the biological response to PAF (Pinckard *et al.*, 1988). In non-excitable cells, such as epithelial cells, Ca^{++} is mobilized by at least 2 different mechanisms. Plasma-membrane receptors allow influx of Ca^{++} from the external environment by opening receptor-operated Ca^{++} channels (Petersen, 1990). Recent evidence using agonist stimulated patch-clamped epithelial cells has suggested a role for voltage-gated Ca^{++} channels in this process as well (Hescheler *et al.*, 1988; Velasco *et al.*, 1988). Inositol 1,4,5-trisphosphate (IP3) is a second messenger that releases Ca^{++} from internal reservoirs such as the endoplasmic reticulum (Berridge and Irvine, 1989) and the calciosome (Volpe *et al.*, 1988). The endoplasmic reticulum has a high affinity IP3 receptor that is linked to Ca^{++} escape from the endoplasmic reticulum (Berridge adn Irvine, 1989).

We postulated that PAF has a direct effect on epithelial cells through a receptor operated calcium messenger system and we tested this possibility in a cultured epithelial cell line, Madin-Darby Canine Kidney (MDCK). A calcium sensitive fluorescent dye, fura-2, was used to measure the biological response of the cells to stimulation with PAF.

Materials and Methods

Materials

Madin-Darby Canine Kidney (MDCK) cells were purchased from the American Type Culture Collection (Rockville, MD.). Medium 199 (M199), heat inactivated fetal bovine serum (FBS), penicillin, streptomycin, gentamycin, bovine serum albumin (BSA), type VII rat tail collagen and nifedipine were obtained from Sigma (St. Louis, MO.). Ionomycin was purchased from Terochem Laboratories (Edmonton, AB.). 35 mm plastic culture plates, 25 mm diameter round glass coverslips (number 2 thickness) were purchased from Fisher (Edmonton, AB.). Platelet-activating factor (1-alkyl-2-acetoyl-*sn*-glycero-3-phosphocholine, PAF) and lyso-PAF (1-alkyl-2-hydroxyl-*sn*-glycero-3-phosphocholine) were bought from Avanti Polar Lipids (Alabaster, AL). WEB 2086 was a gift from Boehringer Ingelheim (Ridgefield, CT.). Fura-2 acetoxymethyl ester (Fura-2 AM) was obtained from Molecular Probes (Eugene, OR.)

Cell Culture

MDCK cells, between passage 55 to 68, were cultured in M199 supplemented with 10% FBS, 60 μg/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin on 35 mm plastic culture dishes and incubated at 37° C in 5% CO_2 and 95% air. The medium was changed every 2 to 3 days. For passage, the cells were exposed to 2 ml of 50 mg% trypsin and 20 mg% EGTA in calcium-free and magnesium-free Hanks Balanced Salt Solution (HBSS) for 5-15 minutes. The cells were then plated on 25 mm diameter round glass coverslips coated with 35.7 $\mu g/cm^2$ type VII rat tail collagen. The

coverslips with the cells attached were used for $[Ca^{++}]i$ measurements within 48-96 hours.

Intracellular Ca^{++} Measurements

All $[Ca^{++}]i$ measurements were done in HEPES buffer containing (in mM): NaCl 130; KCl 3.5; $MgCl_2$ 1.0; $CaCl_2$ 1.0; KH_2PO_4 1.5; HEPES 20; glucose 10, at pH 7.4. Experiments to examine the role of extracellular calcium on cellular response used the above buffer except that $CaCl_2$ was omitted and replaced with 2 mM EGTA. The MDCK cells were incubated in M199 containing 7.5 μM fura-2 AM for 1 hour at 37° C in 5% CO_2 and 95% air (McCoy *et al.*, 1988). Fura-2 AM was prepared as a 1 mM stock solution in DMSO. After loading, the cells were washed to remove excess fura-2 AM. The coverslip was then mounted in a Sykes-Moore chamber designed for a microscope platform and 1 ml of HEPES buffer was added. The cells were allowed to equilibrate at room temperature and room air for 10 minutes. The volume within the Sykes-Moore chamber was maintained at 1 ml. A Fluoroplex III spectrofluorometer (Tracor Northern; Middleton, WI.) and a Nikon Diaphot inverted microscope were used for fluorescence measurements (figure 1). Excitation wavelengths of 340 nm and 380 nm were used. The emission wavelength was 510 nm. Fluorescence readings were recorded for 3 minutes for each PAF experiment. The first 10 seconds of each experiment was used as a baseline measurement before the PAF was delivered to the cells. $[Ca^{++}]i$ was calculated using the standard formula described by Grynkiewicz *et al.* (Grynkiewicz *et al.*, 1985):

$$[Ca^{++}]i = Kd\ [(R-Rmin)/(Rmax-R)][Sf2/Sb2].$$

The Kd of fura-2 was assumed to be 224 nM (Grynkiewicz *et al.*,

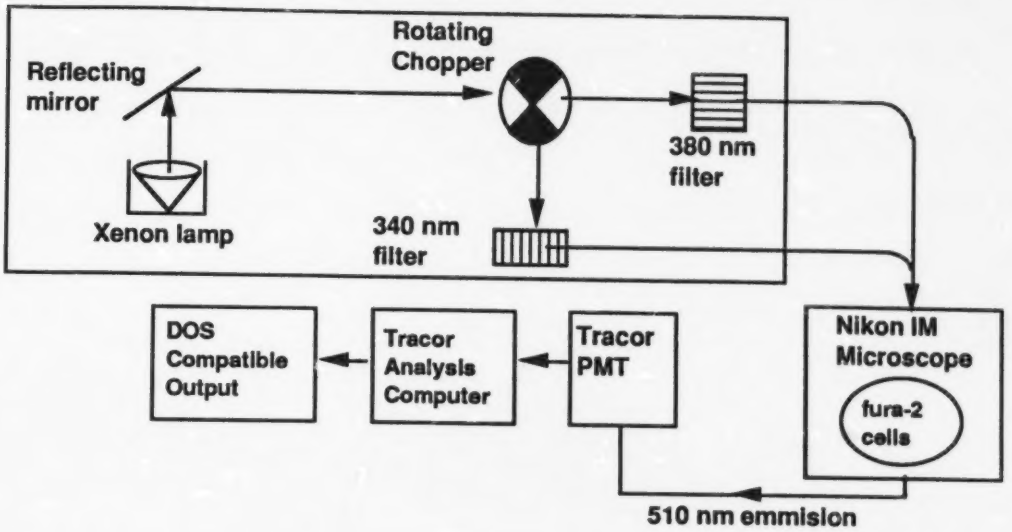

Figure 1. Fluoroplex III Spectrofluorometer Schematic. The xenon lamp output is split by a rotating chopper (0.5 rev./sec.) and passed through a 340 or 380 nm filter. The resulting light is transmitted through a Nikon diaphot fluorescence microscope to the fura-2 loaded cells. Fura-2 emitted light is then passed to a photomultiplyer tube and a computerized analysis unit.

1985). R is the ratio of 340 nm excitation over 380 nm excitation at 510 nm emission. Rmax was determined by first treating the cells with 10 μM ionomycin in the presence of 1 mM extracellular calcium (Tsien, 1989; Williams and Fay, 1990). The Rmin was then calibrated by adding 20 mM EGTA (Scanlon *et al.*, 1987; Williams and Fay, 1990). Sf2 is the 380 nm intensity counts at Rmin (unbound fura-2), Sb2 is the 380 nm fluorescence at Rmax (calcium bound fura-2). Autofluorescence was measured using cells grown on the same coverslips but not loaded with fura-2 and was automatically subtracted from all fluorescent intensity measurements.

Dose Response to PAF

PAF was reconstituted in HEPES buffer + 0.01% BSA (vehicle) to make a $2x10^{-4}$ M stock solution. BSA was used to stabilize PAF and to act as a carrier protein. The stock solution was then diluted in HEPES buffer + 0.01% BSA to create bath concentrations of PAF ranging from 10^{-5} M to 10^{-8} M. Lyso-PAF was prepared in the vehicle as a $2x10^{-4}$ M stock solution and used at a final bath concentration of 10^{-5} M. Both the vehicle and lyso-PAF were studied as controls. A Hamilton syringe was used to deliver the drug. Only one concentration of PAF was tested per plate in order to avoid tachyphylaxis. From the dose response curve, 10^{-5} M PAF was chosen for the remaining experiments as this concentration yielded a sub-maximal biological response and avoided any possible non-specific effects of PAF.

Extracellular Ca^{++} Contribution to PAF Response

To study the role of extracellular calcium on the biological response of epithelial cells to PAF stimulation, fura-2 loaded MDCK cells were also equilibrated for 10 minutes in Ca^{++}-free HEPES buffer ($CaCl_2$ was omitted and replaced with 2 mM EGTA) before 10^{-5} M PAF stimulation. PAF used to stimulate the cells in the Ca^{++}-free HEPES buffer was prepared using Ca^{++}-free HEPES buffer + 0.01% BSA. Fluorescence intensity changes were monitored as above.

Effect of Nifedipine and WEB 2086 on the $[Ca^{++}]i$ Response to PAF

In order to study the nature of Ca^{++} mobilization induced by PAF, two types of blockers were employed. Nifedipine (a dihydropyridine blocker of voltage dependent Ca^{++} channels), and WEB 2086 (a PAF receptor blocker) were tested, both individually and together, for

inhibitory effects on the epithelial cell response. In these experiments, 10^{-5} M nifedipine (McCoy *et al.*, 1988) was added 10 minutes before PAF stimulation and 10^{-5} M WEB 2086 (Rieves *et al.*, 1992) was added 1 minute prior to PAF stimulation. These same conditions were maintained when nifedipine and WEB 2086 were tested together.

Statistics

Data are presented as means ± SEM. Statistical comparison between baseline $[Ca^{++}]i$ and peak $[Ca^{++}]i$ response was done by paired t-test. One-way ANOVA was used to compare the means of different experimental groups. Significance was defined as $p \leq 0.05$.

Results

Effect of PAF on $[Ca^{++}]i$

Epithelial cells showed a dose dependent response to PAF. Figure 2 shows the baseline $[Ca^{++}]i$ before PAF stimulation and the peak $[Ca^{++}]i$ in response to PAF. In general, $[Ca^{++}]i$ rose quickly upon the addition of PAF, peaking within the first 60 seconds then dropping to a level that was above the original baseline (figure 3). All concentrations of PAF significantly increased $[Ca^{++}]i$ from the baseline value ($p<0.05$), except for the lowest dose, 10^{-8} M. The baseline values remained stable throughout the experiments. The dose response data was plotted on a % response versus log dose curve and, from this graph, the apparent EC50 value for PAF effect was determined to be 5.8×10^{-6} M (Fingl, 1975). The vehicle control (HEPES buffer + 0.01% BSA) and 10^{-5} M lyso-PAF did not affect $[Ca^{++}]i$ levels in MDCK cells.

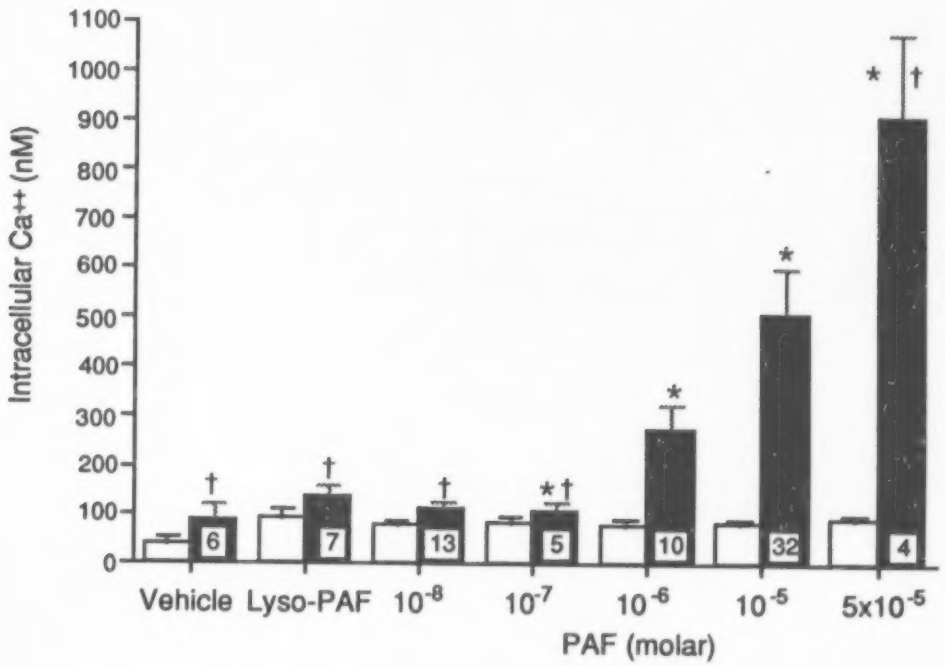

Figure 2. Dose Response of [Ca++]i to PAF stimulation in MDCK cells.

These data show the effect of increasing concentrations of PAF as well as the vehicle and lyso-PAF (10^{-5} M). Open bars (□) are the baseline [Ca++]i values before stimulation. Solid bars (■) are the peak [Ca++]i responses. Numbers inside the solid bars are the n for each test. Values are means ± SEM for individual monolayers (i.e. not for cumulative addition). * indicates $p<0.05$ (by paired t-test) for the peak [Ca++]i versus the respective baseline. † indicates $p<0.05$ (by one-way ANOVA) for peak [Ca++]i versus 10^{-5} M peak [Ca++]i. Apparent EC50 = 5.8×10^{-6} M.

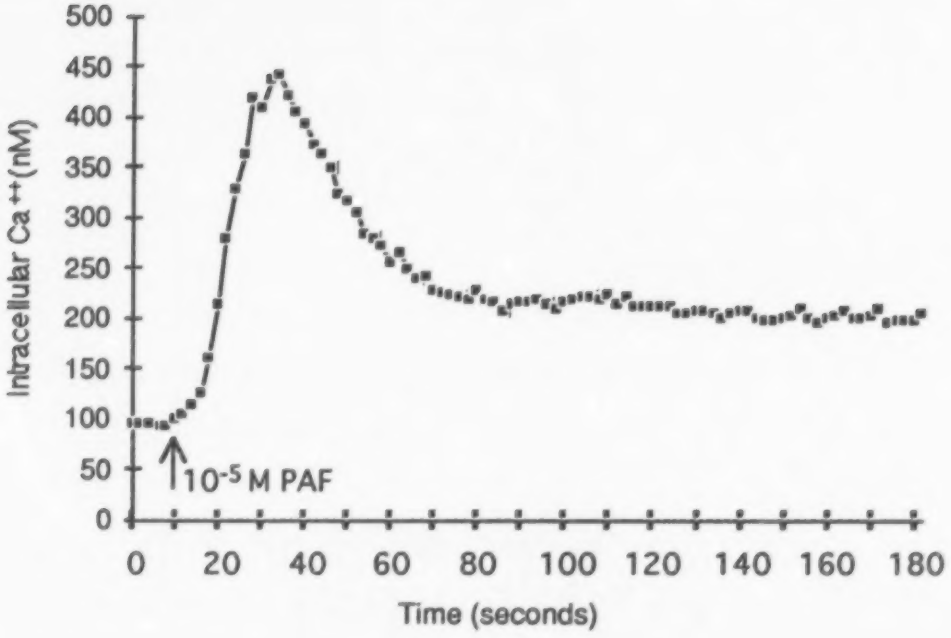

Figure 3. Changes in [Ca++]i to PAF stimulation.
10^{-5} M PAF was added after approximately 10 seconds of baseline measurement. The total recording time was 180 seconds. Peak [Ca++]i response was generally achieved within the first minute after which the [Ca++]i declined to a level above the original baseline for the remainder of the experiment.

Effect of Reducing Extracellular Ca^{++} on $[Ca^{++}]i$ Response to PAF

Replacing the extracellular $[Ca^{++}]$ with 2 mM EGTA resulted in a 75% decrease in the baseline $[Ca^{++}]i$ (figure 4) indicating that treatment of the cells with EGTA could, at least partially, have depleted the intracellular Ca^{++} ion stores. Stimulation of MDCK cells with 10^{-5} M PAF in 1 mM extracellular Ca^{++} raised the $[Ca^{++}]i$ from 84.18 ± 9.60 nM to 500.68 ± 97.48 nM ($n=32$, $p<0.0001$). In the absence of extracellular Ca^{++}, PAF stimulation raised the $[Ca^{++}]i$ from 20.80 ± 3.61 nM to 113.38 ± 30.18 nM ($n=10$, $p<0.05$). This indicates that when extracellular Ca^{++} is removed, the response to PAF is decreased by 77%. The response under reduced extracellular Ca^{++} accounts for approximately 23% of the total increase in $[Ca^{++}]i$ to PAF stimulation.

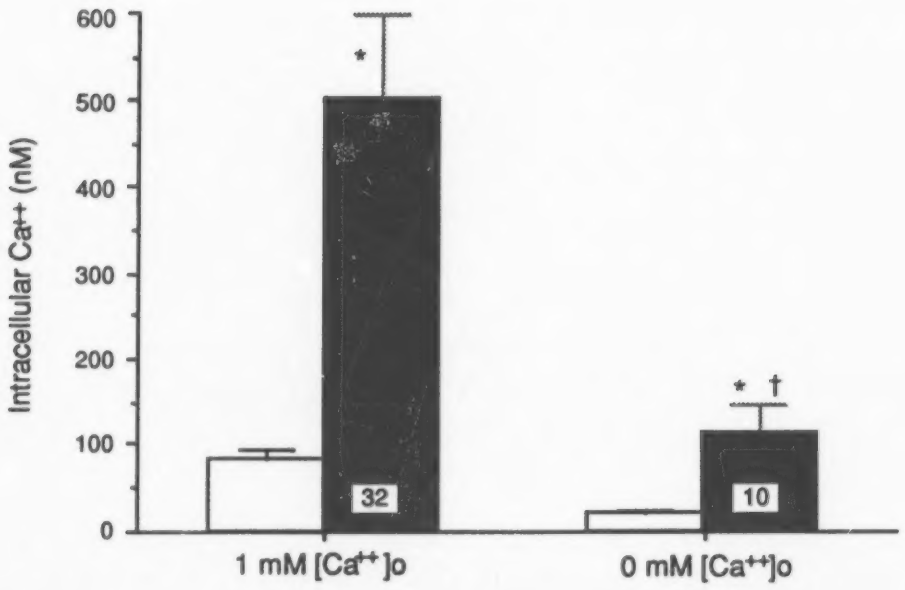

Figure 4. Effect of extracellular calcium on the response to 10^{-5} M PAF stimulation.
To individual monolayers, 2 mM EGTA replaced 1 mM $CaCl_2$ 10 minutes before PAF stimulation. Open bars (□) are the baseline $[Ca^{++}]i$ values. Solid (■) bars are the peak $[Ca^{++}]i$ responses to PAF stimulation. Values are means ± SEM for individual monolayers. The n is indicated by the number in the solid bar. * indicates $p<0.05$ for peak response versus the respective baseline; † indicates $p<0.05$ for peak versus peak comparison.

Effect of PAF Blockers on Ca^{++} Flux due to PAF

The effects of 10^{-5} M nifedipine (McCoy *et al.*, 1988), 10^{-5} M WEB 2086 (Rieves *et al.*, 1992) and the combination of nifedipine and WEB 2086 on $[Ca^{++}]i$ in MDCK cells are shown in figure 5. In the nifedipine treated cells, the $[Ca^{++}]i$ rose 78% ($p<0.05$, $n=8$) above baseline which is a 74% decrease in the peak $[Ca^{++}]i$ response to 10^{-5} M PAF stimulation. The small increase in the nifedipine baseline value was due to the slight fluorescent properties of nifedipine at this concentration (McCoy *et al.*, 1988). In cells treated with the specific PAF receptor blocker WEB 2086, the $[Ca^{++}]i$ increased 206% from baseline ($p<0.05$, $n=12$) or, a 64% decrease in peak $[Ca^{++}]i$ response from peak control values. When used together, nifedipine and WEB 2086 decreased the PAF response by 95%. After subtracting the baseline $[Ca^{++}]i$ from the peak $[Ca^{++}]i$ response, the WEB + nifedipine response to PAF stimulation was significantly less than the control response ($p<0.05$ by one-way ANOVA).

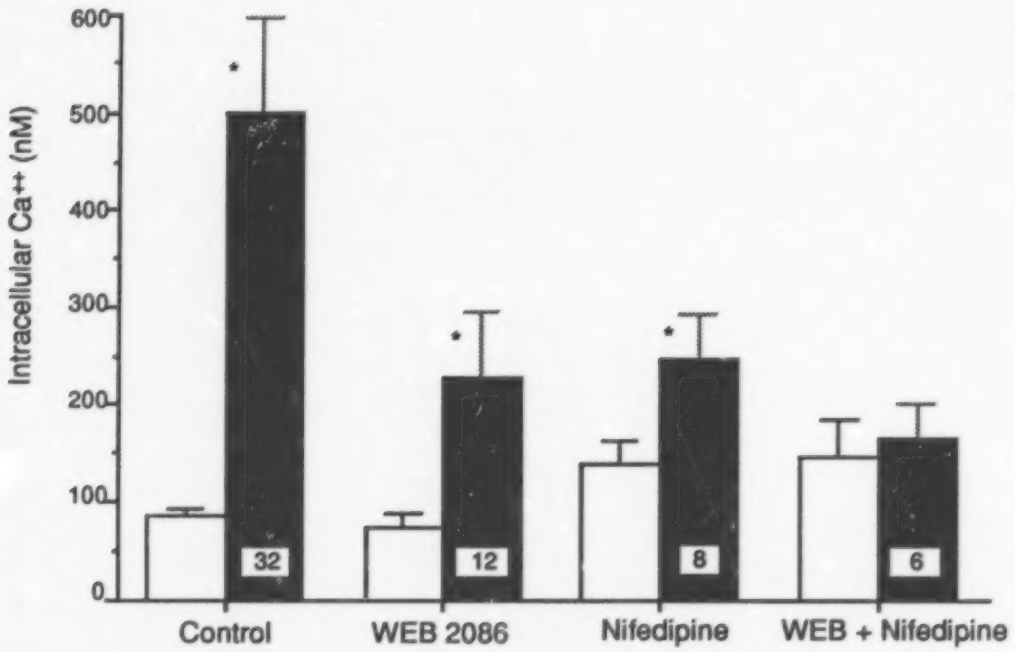

Figure 5. Effect of WEB 2086 and Nifedipine on PAF response.

10^{-5} M WEB 2086 was added 1 minute before PAF stimulation. Cells were incubated for 10 minutes with 10^{-5} M nifedipine before PAF addition. These conditions were maintained when both blockers were used in combination. All experiments were done on individual monolayers. Open bars (□) are the baseline $[Ca^{++}]i$ values. Solid bars (■) are peak $[Ca^{++}]i$ responses to PAF stimulation. Values are the means ± SEM for individual monolayers. The number inside the solid bar is the n for each test. * indicates $p<0.05$ for peak responses versus the respective baseline.

Discussion

PAF is a potent inflammatory mediator that has a wide range of activities and a diverse population of responsive cell types. PAF can induce an increase in the $[Ca^{++}]i$ through activation of calcium channels and through mobilization of intracellular Ca^{++} stores via IP3 (Pinckard *et al.*, 1988). In epithelial cells, PAF affects transepithelial permeability, prostaglandin synthesis (Churchill *et al.*, 1991; Kawaguchi and Yasuda, 1986), phospholipase C activity (Kawaguchi and Yasuda, 1986), bioelectric properties (Tamaoki *et al.*, 1991; Widdicombe *et al.*, 1991) and mucous secretion (Rieves *et al.*, 1992). In this study we investigated the $[Ca^{++}]i$ changes in MDCK epithelial cells upon stimulation with PAF.

At micromolar concentrations, PAF is reported to have detergent properties that may affect the cellular response (Sawyer and Andersen, 1989). The effects of 10^{-5} M PAF on MDCK cells are not due to these proposed detergent properties. The reasons for this are 3 fold: firstly, the intensity counts of the fluorescent probe fura-2 remained stable throughout the experiment indicating that the cell membrane integrity was not disrupted and fura-2 could not diffuse out from the field of view; secondly, the structurally similar but biologically inactive lyso-PAF would be expected to disrupt the cell membrane integrity in a similar manner, this is seen neither in $[Ca^{++}]i$ rise nor in decreased fura-2 intensity counts; finally, the detergent effects of PAF were seen only at 10^{-4} M PAF, which caused an immediate spike in the intensity counts followed by rapid decrease in both 340 and 380 nm intensity counts. This type of response indicates that cell membrane disruption is severe enough to

allow fura-2 to diffuse out of the cytoplasm and away from the field of view. These effects are not seen in MDCK cells and demonstrate that the PAF response is mediated through a specific membrane receptor.

Fura-2 calibration was done *in situ* using ionomycin and EGTA. While there are some questions about this method (Scanlon *et al.*, 1987; Tsien, 1989; Williams and Fay, 1990), the intracellular calcium concentrations that were calculated are in close agreement with the expected values. Baseline $[Ca^{++}]i$ values calculated here are slightly less that the value for MDCK cells reported by McCoy (McCoy *et al.*, 1988) but are still within the range. The main problem with this method of calibration is a possible underestimation of $[Ca^{++}]i$ due to incomplete fura-2AM hydrolysis or sequestration of fura-2AM into intracellular organelles; this does not appear to be a factor in this study.

The results presented here are consistent with the hypothesis that activation of the epithelial cell PAF receptor opens a Ca^{++} channel that allows influx of extracellular Ca^{++} and initiates a mechanism that mobilizes intracellular Ca^{++} stores. Calcium entry appears to be mediated, at least in part, by L-type voltage-sensitive Ca^{++} channels; these channels are inhibited by dihydropyridine compounds like nifedipine. In order to more fully examine the role that nifedipine-sensitive channels play in the PAF response, a dose-response curve to nifedipine would be necessary in order to show that complete inhibition of Ca^{++} channels was obtained. The diminished response when the extracellular Ca^{++} was removed further suggests a role for extracellular Ca^{++} in the PAF response.

Intracellular Ca^{++} stores are possibly mobilized via the phosphoinositol cascade (Berridge and Irvine, 1989). PAF stimulation has been shown, in human platelets, to activate the phosphatidylinositol pathway, raise $[Ca^{++}]i$, cause protein kinase C translocation, and protein phosphorylation (Block *et al.*, 1989). It is believed that this mechanism of action also exists in epithelial cells.

PAF stimulates MDCK cells in a dose dependent manner. The apparent EC50 value for PAF stimulation is $5.8x10^{-6}$ M. This concurs with the value of $3.5x10^{-6}$ M reported for the response in NG 108-15 neuroblastoma/rat glioma cell line using a similar experimental protocol (Kornecki and Ehrlich, 1988). However, the value differs from the EC50 value of 11.5 nM for the response of guinea pig eosinophils (Kroegel *et al.*, 1989). The reason for the large discrepancy is not clear at this time, however, there are some possible explanations. The higher EC50 values were reported from cancer derived cell lines, where the receptor characteristics or intracellular mechanisms inherent to cell lines may deviate from those of normal primary cells (Hwang, 1990). In addition, there could be differences in receptor populations on the cell lines.

Various types of blockers attenuate the biological response to PAF. Nifedipine reduced the peak PAF response in epithelial cells. It appears that dihydropyridine-sensitive Ca^{++} channels are activated by the PAF receptor mechanism. In adrenal cortical cells and in insulin-secreting pancreatic acinar cells, there is apparent second messenger recruitment of voltage-dependent channels in the response to hormone stimulation (Hescheler et al., 1988; Velasco *et al.*, 1988). A similar mechanism may be operating in MDCK cells. The

PAF response was diminished with WEB 2086, a known PAF receptor blocker. In airway epithelial cells 10^{-5} M WEB 2086 caused complete inhibition of an equimolar amount of PAF (Rieves *et al.*, 1992). This difference could be due to PAF receptor heterogeneity between these two epithelial cell types or to species differences (Hwang, 1990). The combination of WEB 2086 and nifedipine completely inhibited the response to PAF. This apparent co-operative inhibition of the PAF response suggests the presence of two distinct entities on the plasma membrane for these two compounds. Complete dose response curves for nifedipine and WEB 2086 are necessary to determine their efficacy and potency in these cells and in order to ensure that complete inhibition was accomplished with the chosen concentrations.

When the extracellular Ca^{++} was reduced to less than 1 nM a significant reduction in baseline $[Ca^{++}]i$ was seen. A similar response occurred in a previous study using MDCK cells (McCoy *et al.*, 1988). The decrease in the baseline was possibly due to a concentration gradient that made Ca^{++} higher in the cytoplasm than the surrounding medium. This had the effect of reducing the basal $[Ca^{++}]i$ level perhaps by enhancing Na^{+}/Ca^{++} co-transporter function or perhaps by depleting the intracellular stores of Ca^{++}. The large decline in the PAF response when extracellular calcium was removed and the reduced reponse in the presence of nifedipine suggests that the PAF response is highly, but not exclusively, dependent upon the influx of Ca^{++} from the extracellular milieu.

In conclusion, our results indicate that PAF has a direct effect on the intracellular calcium levels in epithelial cells and that this

effect is mediated through a PAF specific receptor. PAF elevates $[Ca^{++}]i$ in MDCK epithelial cells in a dose dependent manner. The response to PAF stimulation could be reduced by 2 different types of blockers: nifedipine and WEB 2086. The combination of WEB 2086 and nifedipine completely blocked the response to PAF. PAF effect on MDCK cells was largely but not entirely dependent upon extracellular Ca^{++}.

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Chapter 3

PAF Effect on $[Ca^{++}]i$ in Human Nasal Epithelial Explants

Introduction

Identified in 1979 as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, platelet-activating factor (PAF) is produced by many cell types including airway epithelial cells (Pinckard *et al.*, 1988; Holtzman *et al.*, 1991; Salari and Wong, 1990). It is one of the most potent inflammatory mediators known and has been implicated in pulmonary inflammatory diseases including asthma (Hensen, 1989). PAF is produced and released from airway epithelial cells (Holtzman *et al.*, 1988; Salari and Wong, 1988) and, also, it elicits a number of physiological effects on airway epithelial cells such as increased short-circuit current and decreased resistance in epithelial monolayers (Tamaoki *et al.*, 1991; Widdicombe *et al.*, 1989), increased production of prostaglandins PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and thromboxane B_2 (Churchill *et al.*, 1991; Widdicombe *et al.*, 1989), and increased airway mucus secretion (Reives *et al.*, 1992).

In many physiological responses calcium ions are intracellular second messengers and calcium mobilization may be an important element in the biological response to PAF stimulation (Pinckard *et al.*, 1988). In non-excitable cells, such as epithelial cells, Ca^{++} is mobilized through plasma-membrane receptor-operated channels that open upon stimulation to allow the influx of extracellular Ca^{++} (Petersen, 1990). Voltage-gated L-type Ca^{++} channels may also play a role in this process although the mechanism of their involvement is uncertain at this time (Heschler *et al.*, 1988; Velasco *et al.*, 1988). Ca^{++} is also mobilized from intracellular stores such as endoplasmic reticulum (Berridge and Irvine, 1989) and the calsiosome (Volpe *et*

al., 1988) by intracellular messengers such as inositol 1,4,5-trisphosphate.

The physiological effects of PAF are well characterized in many cell types but the cellular mechanism of action of PAF in airway epithelial cells remains to be established. In many cells including macrophages (Conrad and Rink, 1986), platelets (Pinckard *et al.*, 1988), kidney epithelial cells (Ueda *et al.*, 1991) and MDCK cells (see chapter 2), PAF increases the intracellular calcium ion concentration, $[Ca^{++}]i$, by a receptor-mediated process. Increasing the $[Ca^{++}]i$ could be a potential mechanism by which PAF could exert its effects. Radio-ligand binding studies have identified PAF receptor sub-types on a number of different cell types (Hwang, 1990). The recent cloning of the PAF receptor has confirmed that the PAF receptor belongs within the family of G-protein binding receptors (Honda *et al.*, 1991; Shukla, 1992). These PAF receptors activate, via G-proteins, the phosphoinositol pathway and increase $[Ca^{++}]i$ by allowing an influx of extracellular Ca^{++} and by releasing Ca^{++} from intracellular stores (Pinckard *et al.*, 1988). The mechanisms and characteristics of the action of PAF that have been established in other cell types could also be important in understanding the response to PAF in airway epithelial cells.

We hypothesized that PAF acts directly on airway epithelial cells through a specific membrane receptor and activates a cascade mechanism that leads to an increase in $[Ca^{++}]i$. This hypothesis was tested using cultured airway epithelial cells and the calcium sensitive fluorescent dye, fura-2. The cells were cultured from explants to preserve the heterogenous nature of the native respiratory

epithelium. We also examined the effects of selective antagonists to elucidate the mechanism of action of PAF on airway epithelial cells.

Materials and Methods

The use of human tissues was approved by the Research and Ethics Committee of the Faculty of Medicine, University of Alberta, Edmonton, Alberta.

Materials

Chemicals and products were purchased as follows: DME/F12 medium, 10X M199, heat inactivated fetal bovine serum (FBS), penicillin, streptomycin, gentamycin, transferrin, endothelial cell growth supplement, epidermal growth supplement, hydrocortisone, insulin, 3,3',5-triiodo-L-thyronine (T3), rat tail collagen, indomethacin, bovine serum albumin (BSA), phorbol 12-myristate 13-acetate (PMA), and n-ethylmaleimide (NEM) were from Sigma Chemical Co., St Louis, MO; fura-2 acetoxymethyl ester was from Molecular Probes, Eugene, OR.; platelet-activating factor (1-alkyl-2-acetyl-*sn* -glycero-3-phosphocholine, PAF) and lyso-PAF (1-alkyl-2-hydroxyl-*sn* -glycero-3-phosphocholine) were from Avanti Polar Lipids, Alabaster, AL. WEB 2086 was a gift from Boehringer Ingelheim (Ridgefield, CT). Alprazolam was a gift from Upjohn Laboratories (Kalamazoo, MI).

Explant Cell Culture

Round glass coverslips (number 2 thickness) used for explant cell culture were coated under sterile conditions with 175 μg of rat tail collagen and allowed to dry overnight. Surgical specimens were obtained from patients undergoing routine turbinectomy in the day surgery suite. Turbinates, 1 to 4 hours after surgery, were returned to the laboratory where the mucosa was dissected off and cut into tiny explants (approximately 2 mm^2). A collagen solution was

prepared from the stock solutions listed below using a modified procedure of Yang *et al.* (Yang *et al.*, 1981) to anchor the explant to the coverslip. Briefly, 640 µl of a 5 mg/ml rat tail collagen stock solution, 84 µl of 10X concentrated M199 and 94 µl of 0.325 N NaOH stock solution were mixed together to form the collagen-anchor solution. The solution was first placed onto the coverslip and the explant was placed with the mucosal side up immediately afterward. The collagen was allowed to polymerize for 15-30 minutes at 37°C. The explants were cultured in DME/F12 supplemented with 7.5 µg/ml transferrin, 18 ng/ml hydrocortisone, 7.5 µg/ml endothelial cell growth supplement, 13 ng/ml epidermal cell growth factor, 2 µg/ml insulin, 2 ng/ml T3, 60 µg/ml penicillin G, 100 µg/ml streptomycin sulfate and 50 µg/ml gentamycin in 1% (v/v) FBS at 37°C, 5% CO_2, 98% relative humidity (Yankaskas *et al.*, 1985). The culture medium was changed every 2 to 3 days and the cells were used after 7 to 10 days in culture.

Intracellular Ca^{++} Measurements

All $[Ca^{++}]i$ measurements were done in HEPES-buffered Ringers solution that contained (mM): 135 NaCl, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 10 HEPES with pH adjusted to 7.4 (McCann *et al.*, 1990). Those experiments that were to examine the effect of low extracellular calcium concentration on cellular response used the above HEPES buffer but 3 mM EGTA was added 10 minutes prior to PAF stimulation to reduce the free extracellular Ca^{++} level to 40 nM (Stockbridge, 1987). To load the human nasal epithelial cell outgrowth from the explant with fura-2 AM, 10 µM fura-2 AM in HEPES buffer was added for 1 hour at room temperature. These

conditions minimize fura-2 loading into intracellular vesicles while maximizing the amount of free fura-2 in the cytoplasm (Scanlon *et al.*, 1987). After 1 hour, excess fura-2 AM was carefully washed off the cells and the coverslip was mounted in a Sykes-Moore chamber and 1 ml of buffer was added. The cells were allowed to equilibrate at room temperature and room air for 10 minutes. The volume within the chamber was maintained at 1 ml. A Fluoroplex III spectrofluorometer (Tracor Northern; Middleton, WI.) and a Nikon diaphot inverted microscope were used for fluorescence measurements. Excitation wavelengths of 340 nm and 380 nm were used. The emission wavelength was 510 nm. $[Ca^{++}]i$ was calculated using the standard formula described by Grynkiewicz et. al. (Grynkiewicz *et al.*, 1985):

$$[Ca^{++}]i = Kd\ [(R-Rmin)/(Rmax-R)][Sf2/Sb2].$$

The Kd of fura-2 was assumed to be 224 nM (Grynkiewicz *et al.*, 1985). R is the ratio of 340 nm excitation over 380 nm excitation at 510 nm emission. Rmax was determined by first treating the cells with ionomycin in the presence of 1.2 mM extracellular calcium (Tsien, 1989; Williams and Fay, 1990). The Rmin was then calibrated by adding 20 mM EGTA (Scanlon *et al.*, 1987; Williams and Fay, 1990). Sf2 was the 380 nm intensity counts at Rmin (unbound fura-2), Sb2 was the 380 nm fluorescence at Rmax (calcium bound fura-2). Autofluorescence was measured using cells grown on the same coverslips but not loaded with fura-2 and was subtracted from all fluorescent intensity measurements.

Dose Response to PAF

PAF was reconstituted in HEPES buffer + 0.01% BSA (vehicle) to make a $2x10^{-4}$ M stock solution. BSA was used to stabilize PAF and to act as a carrier protein. The stock solution was then diluted in the vehicle to create final bath concentrations of PAF ranging from 10^{-5} M to 10^{-8} M. Lyso-PAF was also prepared in HEPES buffer + 0.01% BSA as a $2x10^{-4}$ M stock solution and used at a final bath concentration of 10^{-5} M. Both the vehicle and lyso-PAF were studied as controls. A Hamilton syringe was used to deliver the drug. To test for possible tachyphylaxis of PAF, a second dose of 10^{-5} M PAF was given 5 to 10 minutes after an initial stimulation. When studying the effects of various compounds on the $[Ca^{++}]i$ response to PAF stimulation, only one concentration of PAF was used per plate in order to avoid tachyphylaxis. From the dose response curve, 10^{-5} M PAF was chosen for the remaining experiments as this concentration yielded a maximal biological response and avoided any possible non-specific effects of PAF.

Extracellular Calcium Contribution to PAF Response

To study the role of extracellular calcium on the biological response of epithelial cells to PAF stimulation, fura-2 loaded human nasal epithelial cells were also equilibrated for 10 minutes in HEPES buffer (40 nM Ca^{++}) before stimulation with 10^{-5} M PAF stimulation which was prepared using 40 nM Ca^{++} HEPES buffer + 0.01% BSA. Fluorescence intensity changes were monitored as above.

Effect of Pathway Modulators on the PAF Response

The following compounds were added to the fura-2 loaded human nasal epithelial cells as aliquots of 10 to 100 times

concentrated stock solutions to produced the final bath concentrations indicated. Only one drug and one stimulation of PAF was used per experiment. Two PAF receptor blockers, WEB 2086 and alprazolam, were used to examine the specificity of the response to PAF stimulation. The blockers were used individually at 10^{-5} M and were added to the cells 1 minute prior to PAF stimulation (Rieves *et al.*, 1992). Indomethacin was used to examine the possible modulation of the PAF response by cyclooxygenase products. Indomethacin (10^{-6} M) was incubated with the cells for 15 minutes before PAF stimulation (Widdicombe *et al.*, 1989). N-ethylmaleimide (NEM) was used to examine the role of G-proteins in the PAF response (Fredholm *et al.*, 1985). There is some controversy surrounding the sensitivity of the PAF receptor G-protein to cholera and pertussis toxins (Pinckard *et al.*, 1988); therefore, we chose to examine the G-protein sensitivity to NEM. 10^{-4} M NEM was incubated with the cells for 15 minutes prior to PAF stimulation. Phorbol 12-myristate 13-acetate (PMA) was used to examine the role of activated protein kinase C on the PAF response. 10^{-7} M PMA was incubated with the cells for 30 minutes before PAF stimulation.

Statistics

Data are presented as means ± SEM. Statistical comparison between baseline $[Ca^{++}]i$ and peak $[Ca^{++}]i$ response was done by paired t-test. One-way ANOVA was used to compare the means of different experimental groups. Significance was defined as $p \leq 0.05$.

Results

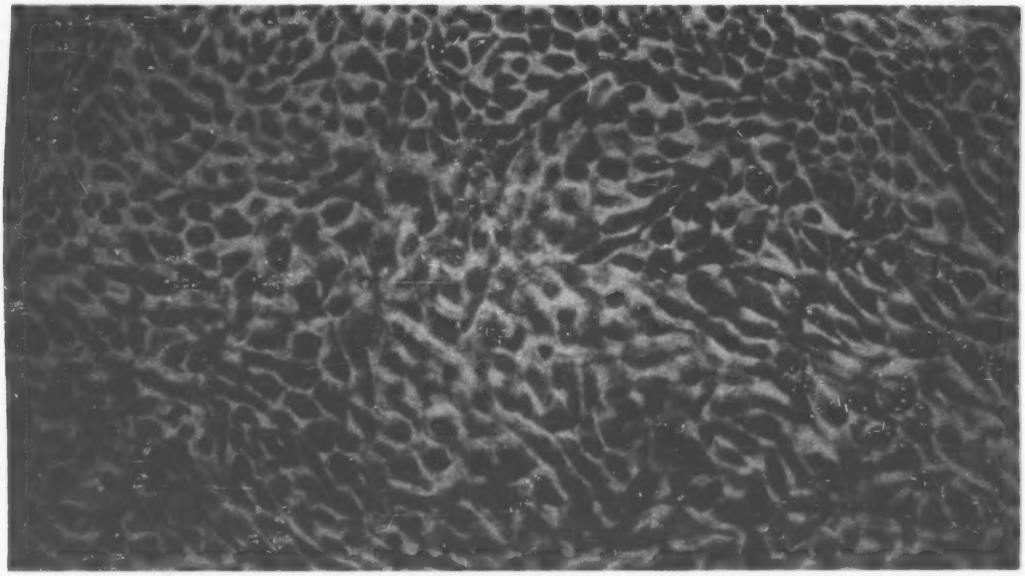
Epithelial cell culture

The explants were cultured for 7 to 10 days to allow for sufficient out-growth of cells before use. When examined by phase-contrast light microscopy, the outgrowth had cuboidal shaped cells with a cobblestone appearance typical to that of epithelial cells (figure 1a to d). Immunohistochemical studies showed a high degree of staining for keratin, a cytoskeletal protein expressed in epithelial cells and very little anti-vimentin staining, a cytoskeletal protein expressed by fibroblasts, confirming the epithelial nature of the explant outgrowth (Gruenert *et al.*, 1990).

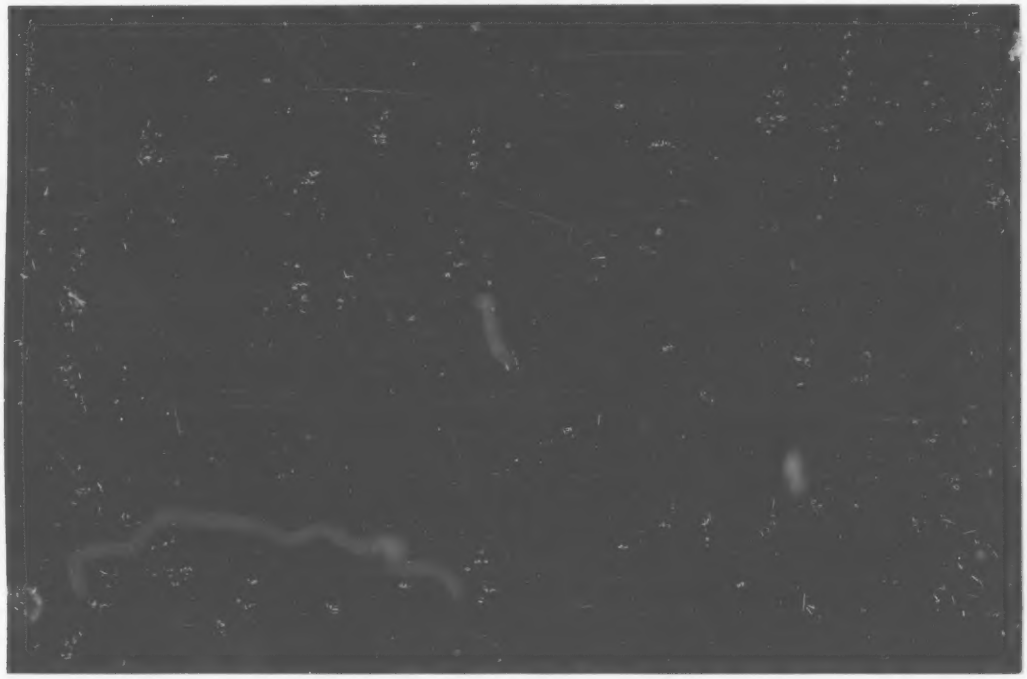
Effect of PAF on $[Ca^{++}]i$

Human nasal epithelial cells responded in a dose dependent manner to PAF stimulation (figure 2). In general, the $[Ca^{++}]i$ rose upon the addition of PAF, peaked within 1 to 3 minutes and stayed at an elevated plateau for at least 5 minutes post-stimulation (figure 3). All concentrations of PAF significantly increased $[Ca^{++}]i$ from the baseline value ($p<0.05$), except for the lowest dose, 10^{-8} M. Stimulation of human nasal epithelial cells with 10^{-5} M PAF elevated $[Ca^{++}]i$ from 73.38 $\pm$ 12.16 nM to 646.20 $\pm$ 127.24 nM (n=14), an increase of 780% above baseline. The vehicle control (HEPES buffer + 0.01% BSA) had a slight but statistically significant effect increasing the $[Ca^{++}]i$ by 106% above baseline ($p<0.05$, n=6). However, 10^{-5} M lyso-PAF did not affect $[Ca^{++}]i$ levels (data not shown).

Figure 1. Photographs of human nasal epithelial cells in explant culture.



1a. Bright field view of epithelial cells.



1b. Fluorescent view of fura-2 loaded epithelial cells.

1c. Immunohistochemical staining of epithelial cells for keratin.

1d. Immunohistochemical staining of epithelial cells for vimentin.

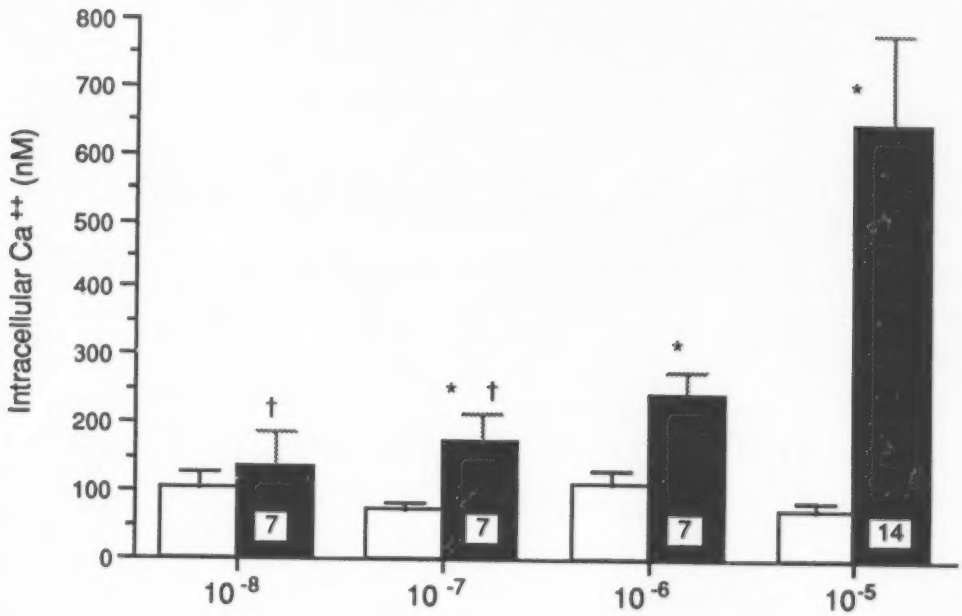


Figure 2. Dose response of [Ca++]i to PAF stimulation. Explants were cultured as described and stimulated with PAF ranging in concentration from 10^{-8} M to 10^{-5} M. Only one dose of PAF was used per plate. Cells in the out-growth responded to PAF stimulation in a dose dependent manner. Results are presented as the mean ± SEM. Open bars (□) are baseline values while solid bars (■) represent peak [Ca++]i values. * indicates $p<0.05$ for peak [Ca++]i versus respective baseline; † indicates $p<0.05$ for peak [Ca++]i versus 10^{-5} M PAF peak [Ca++]i.

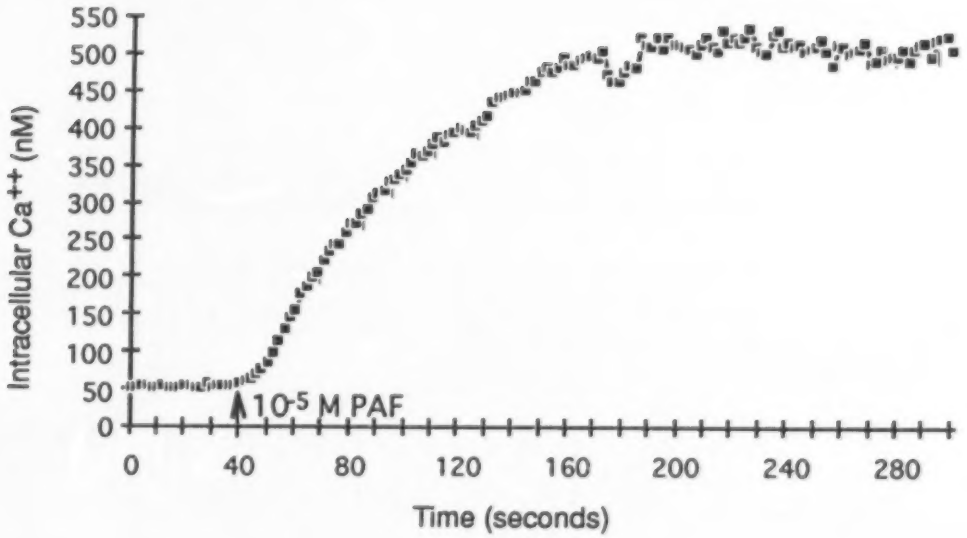


Figure 3. Changes in [Ca++]i to PAF stimulation. 10^{-5} M PAF was added after approximately 40 seconds of baseline measurement. The total recording time was 300 seconds. Peak [Ca++]i response was generally reached within 2-3 minutes and stayed elevated for the remainder of the experiment.

Role of Extracellular Ca^{++} on $[Ca^{++}]i$ Response to PAF

To establish the contribution of extracellular Ca^{++} influx to the PAF response, the extracellular $[Ca^{++}]$ was reduced with 3 mM EGTA. The free extracellular Ca^{++} concentration under these conditions was approximately 40 nM (Stockbridge, 1987). This reduction in extracellular $[Ca^{++}]$ had no effect on the baseline $[Ca^{++}]i$ (Table 1). Stimulation of human nasal epithelial cells with 10^{-5} M PAF in 1.2 mM extracellular Ca^{++} raised the $[Ca^{++}]i$ by 780% ($p<0.001$). When EGTA was added to reduce the free extracellular Ca^{++}, 10^{-5} M PAF stimulation raised the $[Ca^{++}]i$ from 65.46 ± 8.26 nM to 245.02 ± 39.09 nM, an increase of 274% ($p<0.0005$). This response to PAF stimulation represents a 69% decrease in maximal $[Ca^{++}]i$ when extracellular Ca^{++} is reduced ($p<0.05$).

Effect of PAF Receptor Blockers on $[Ca^{++}]i$

PAF receptor blockers 10^{-5} M WEB 2086 (Rieves *et al.*, 1991) or 10^{-5} M alprazolam were added 1 minute before 10^{-5} M PAF stimulation (figure 4) with only one blocker used per experiment. The addition of these compounds to the bathing medium had no effect on the baseline $[Ca^{++}]i$ values. WEB 2086 and alprazolam reduced the response to 10^{-5} M PAF stimulation by 74% and 43% respectively; the peak $[Ca^{++}]i$ for WEB 2086 was significantly reduced compared to 10^{-5} M PAF alone ($p<0.05$).

Effect of Pathway Modulators on the PAF Response

In order to study the mechanisms of the cellular response to PAF, several compounds that activate or inhibit specific cellular pathways were employed to determine their effect on PAF stimulation. These results are shown in figure 5. Both 10^{-4} M NEM

and 10^{-7} M PMA decreased the peak $[Ca^{++}]i$ response to 10^{-5} M PAF stimulation while indomethacin further increased the response.

	Baseline $[Ca^{++}]i$	Peak $[Ca^{++}]i$	% $[Ca^{++}]i$ increase
40 nM Ca^{++}	65.46 ± 8.26	245.02 ± 39.09 * †	274.32
1.2 mM Ca^{++}	73.38 ± 12.18	646.20 ±134.51 *	780.57

Table 1. Contribution of extracellular Ca^{++} to PAF response. 3 mM EGTA was added to the HEPES buffer 10 minutes before 10^{-5} M PAF stimulation. Results are presented as the mean ± SEM. * indicates $p<0.05$ for peak $[Ca^{++}]i$ versus respective baseline; † indicates $p<0.05$ for comparison of peak $[Ca^{++}]i$ values.

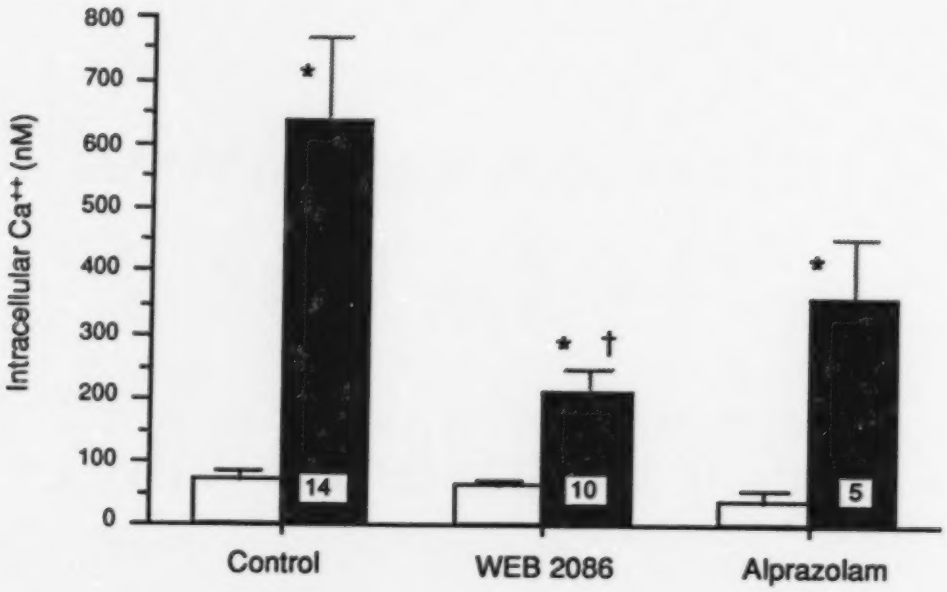


Figure 4. Effect of PAF receptor blockers on [Ca^{++}]i response.
10^{-5} M WEB 2086 or 10^{-5} M alprazolam was added 1 minute prior to 10^{-5} M PAF stimulation. Results are presented as the mean ± SEM. Open bars (□) represent baseline [Ca^{++}]i values while the solid bars (■) represent peak [Ca^{++}]i values to PAF stimulation. * indicates $p<0.05$ for baseline versus respective peak [Ca^{++}]i; † indicates $p<0.05$ for blocker peak [Ca^{++}]i versus control peak [Ca^{++}]i.

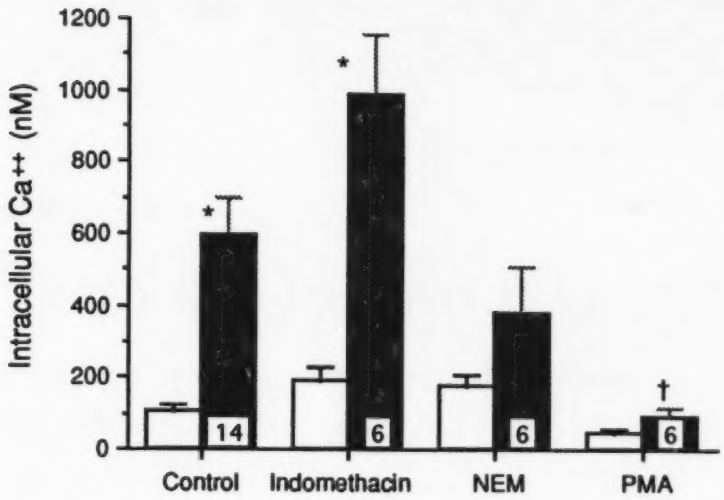


Figure 5. Effect of pathway modulators on [Ca++]i response to PAF stimulation.
Concentrations and incubation times are as indicated in the Methods section. NEM -- N-ethylmaleimide; PMA -- phorbol 12-myristic 13-acetate. Results are presented as the mean ± SEM. Open bars (□) represent baseline [Ca++]i values while the solid bars (■) represent peak [Ca++]i values to PAF stimulation. * indicates $p<0.05$ for baseline versus respective peak [Ca++]i; † indicates $p<0.05$ for peak [Ca++]i of the test compound versus control peak [Ca++]i.

Discussion

We have presented results that indicate that PAF acts on airway epithelial cells through a specific receptor that increases the $[Ca^{++}]i$ and whose function is modulated by G-proteins, protein kinase C and cyclooxygenase products. Although physiological reactions have been documented to PAF stimulation, a mechanism of PAF action has not been previously reported for human nasal epithelial cells.

Explant culture of human nasal epithelium produced an outgrowth rich in epithelial cells and virtually devoid of contaminating fibroblasts as determined by immunohistochemical staining. The cells were used after 7 to 10 days in culture and by this time there were usually few ciliated cells visible by phase-contrast light microscopy.

PAF, at concentrations higher than its critical micelle concentration (CMC) of 10^{-6} M, may have detergent-like effects on the cell membrane that could lead to false readings of physiological response (Sawyer and Andersen, 1989). However, in our cells the PAF response even at 10^{-5} M is specific and appears to be directed by a membrane receptor. Our conclusion is based on the following observations. First, PAF might cause leakiness of the plasma membrane allowing fura-2 to leach from the cytoplasm to the extracellular environment thus reducing fluorescent intensity counts; but, this did not occur. Second, lyso-PAF, a structurally similar but biologically inactive analog of PAF, has no biological effect on the $[Ca^{++}]i$, and third, the responses were reduced by specific PAF receptor antagonists. In addition, the buffer used to reconstitute the

PAF contained 0.01% BSA which may act as a carrier protein and may change the CMC of PAF making this factor less important in these experiments.

The response to PAF in human nasal epithelial cells reached a maximum in 1 to 3 minutes, staying at a plateau level close to the maximum for up to 5 minutes. In other cell types, such as MDCK cells, the response generally reached a maximum in less than 1 minute then fell to a level above the baseline (see chapter 1). The reason for the difference in the Ca^{++} tracing is unknown at this time; however, isolated hepatocytes loaded with fura-2 and stimulated with vasopressin or phenylephrine exhibit similar responses as the human nasal epithelial cells -- rising to a plateau level and remaining at the elevated level for several minutes (Williamson, *et al.*, 1989). These differences with MDCK cells could be due to different receptor densities. This difference in recpetor densities may cause a threshold phenomenon whereby some cells require a longer period of time to aquire enough IP3 to trigger the Ca^{++} response. Alternatively, PAF may require longer diffusion time to reach the cells because of the mucous layer created by mucous secreting cells present in these cultures (Rieves *et al.*, 1992).

Changes in $[Ca^{++}]i$ occurred in a dose dependent manner with 10^{-5} M being the maximal concentration tested. This dose response to PAF in our experiments corresponds to the dose relationship shown for mucus secretion (Rieves *et al.*, 1992) and for alteration of bioelectric properties (Tamaoki *et al.*, 1991). Between 10^{-5} M and 10^{-6} M PAF has been shown to elicit physiological effects such as increased mucus secretion and alteration of short-circuit current and

resistance (Rieves *et al.*, 1992; Tamaoki *et al.*, 1991; Widdicombe *et al.*, 1989). In addition, at 10^{-5} M, in MDCK cells, PAF can stimulate changes in prostaglandin biosynthesis, phospholipase A_2 and C activity (Kawaguchi and Yasuda, 1986) and in $[Ca^{++}]i$ (Labrecque *et al.*, 1991). However, in a pig kidney epithelial cell line (LLC-PK1), the maximal dose required to raise $[Ca^{++}]i$ was 10^{-7} M (Ueda *et al.*, 1991). This discrepancy could be due to receptor heterogeneity between different species, different cell lines, or different cell types (Hwang, 1990).

Extracellular calcium appeared to be responsible for approximately two-thirds of the total change in $[Ca^{++}]i$ due to PAF stimulation, with the remaining one-third presumably coming from internal stores. This dependency on extracellular Ca^{++} has been demonstrated in response to PAF stimulation in other cell types such as macrophages and platelets (Pinckard *et al.*, 1988) as well as in LLC-PK1 pig kidney epithelial cells (Ueda *et al.*, 1991) and MDCK cells (Labrecque *et al.*, 1991). In addition, calcium transport studies in MDCK cells have demonstrated that inositol 1,4,5-trisphosphate released about 25% of accumulated Ca^{++} from endoplasmic reticulum membrane preparations (De Smedt *et al.*, 1988). The value for human nasal epithelial cells shown in our study is in keeping with earlier studies.

The PAF receptor blockers WEB 2086 and alprazolam both inhibited the response to 10^{-5} M PAF stimulation. WEB 2086 appears to be a better PAF receptor inhibitor than alprazolam, decreasing the $[Ca^{++}]i$ response by 74% compared to 43% for alprazolam. This difference could be due to the potency of the drugs at the given

concentrations or to the efficacy of the compounds for binding to the receptor at this concentration. To determine more precisely the inhibitory effects of these two compounds, complete dose response curves would be helpful in determining their potency and efficacy in these cells. WEB 2086 (10^{-5} M) is a potent PAF receptor antagonist blocking a variety of responses in airway epithelial cells from humans, dogs (Tamaoki *et al.*, 1991) and cats (Rieves *et al.*, 1992). The specificity of these PAF inhibitors support the conclusion that the response to PAF is mediated through a specific membrane receptor on the surface of the epithelial cells and not through any possible detergent effect of PAF.

Inhibition of the cyclooxygenase pathway with indomethacin appeared to increase the human nasal epithelial cell response to PAF stimulation. While the change did not reach statistical significance, the PAF response in the presence of indomethacin was 63% greater than without indomethacin present. This response may suggest a modulating role for cyclooxygenase products in the cellular response to PAF. Tamaoki *et al.* (1991) recently demonstrated indomethacin inhibition of short-circuit current increases in response to PAF. However, this result contradicted an earlier study by Widdicombe *et al* (1989) which showed no effect of indomethacin on the short-circuit current increase induced by PAF. In addition, altered arachidonic acid metabolism plays an important role in airway mucus secretion (Rieves *et al.*, 1992). The precise role of cyclooxygenase products on the cellular response to PAF stimulation and the relationship of $[Ca^{++}]i$ to the cellular responses of airway epithelial cells remains to be conclusively elucidated.

The G-protein inhibitor NEM reduced the response to PAF stimulation by 76% and did not increase [Ca^{++}]i significantly from baseline. NEM has been used to block adenosine receptor-mediated cAMP accumulation by inactivating the receptor's nucleotide-binding protein (G-protein) (Fredholm *et al.*, 1985). Our result with NEM suggests that there is interaction between the PAF receptor and a G-protein. This also concurs with the classification of the recently cloned PAF receptor from guinea-pig lungs into the superfamily of G-protein receptors (Honda *et al.*, 1991).

Our results are consistent with the model that PAF activates airway epithelial cells by binding to a specific membrane receptor which activates Ca^{++} channels allowing an influx of extracellular Ca^{++}. The receptor is linked to G-protein transmission of the message to an intracellular signaling mechanism. This intracellular signaling mechanism leads to a release of intracellular Ca^{++} stores and may be involved in regulating extracellular Ca^{++} influx. The increase in the [Ca^{++}]i activates protein kinase C (PKC) which may play a role in regulating the activity of the receptor (Rassmussen, 1989) . Activation of PKC is suspected of desensitizing the PAF receptor probably by phosphorylation. There may be some modulatory interactions in this mechanism by the phospholipase C pathway and by the cyclooxygenase pathway.

In conclusion, we have found that PAF can act directly on human nasal epithelial cells through a specific receptor to increase the [Ca^{++}]i. These observations may be important in understanding the mechanism of interaction between these two elements.

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Chapter 4

Conclusion

The focus of this project was to examine the interaction between platelet-activating factor (PAF) and intracellular calcium ion concentration ($[Ca^{++}]i$) in epithelial cells. This chapter shall discuss, briefly, background elements of these three topics, examine the important findings of the two research studies and state the major conclusions from the project.

PAF is produced by a number of cell types including epithelial cells (Holtzman *et al.*, 1991, Salari and Wong, 1990) and exerts its physiological effects in both a paracrine and an autocrine manner. It is unclear why cells would both produce PAF and be stimulated by it. In epithelial cells, PAF stimulation results in many physiological changes including increased arachidonic acid metabolism, activation of phospholipase A2 and phospholipase C (Kawaguchi and Yasuda, 1986), increased mucus secretion (Reives *et al.*, 1992) and alteration of the short circuit current and transepithelial resistance in cultured monolayers (Tamaoki *et al.*, 1991; Widdicombe *et al.*, 1989).

PAF is a potent inflammatory mediator that has been implicated in a number of pathophysiological responses including hypertension, systemic lupus erythematosus and asthma (Pinckard *et al.*, 1988). The ability of PAF to induce bronchial hyper-responsiveness in humans and other species (Barnes, 1989) is of current interest due to the increase in asthma mortality (Costello, 1991). Bronchial hyper-responsiveness is the hallmark of asthma and the inflammatory response induced by PAF mimics the asthmatic response. While the relationship between PAF, bronchial hyper-responsiveness and asthma is by no means certain, this temporal association indicates that the effects of PAF need to be

investigated to more clearly elucidate its role in inflammatory pathologies.

Intracellular calcium plays a universal role as a second messenger. Alteration of calcium homeostasis results in physiological responses such as mucus secretion (Dobbs *et al.*, 1986) and smooth muscle contraction (Karaki andWeiss, 1988). In epithelial cells, changes in $[Ca^{++}]i$ affect the paracellular permeability of cultured monolayers (Winter *et al.*, 1991), lead to the generation of arachidonic acid metabolites (Henke *et al.*, 1988) and play a role in Cl^- secretion (Hartmann *et al.*, 1992). The normal $[Ca^{++}]i$ in epithelial cells is approximately 10^{-7} M and alteration in the $[Ca^{++}]i$ can be induced by a number of agonists including bradykinin, PAF, calcium ionophore and isoproterenol (Hartmann *et al.*, 1992).

In epithelial cells there are a number of physiological responses common to both PAF and to increased $[Ca^{++}]i$, and these include mucus secretion, arachidonic acid metabolism and Cl^- secretion. It is reasonable to postulate that there exists an intimate connection between the stimulation of the cells with PAF, an alteration in the $[Ca^{++}]i$ and the physiological response.

The epithelium forms a natural barrier in many organs and serves as a liaison between the external and the internal milieu. The airway epithelium is a complex structure. Cell culture techniques that preserve the native epithelial structure should allow for a closer approximation of what happens *in vivo*. Thus, airway explant studies have a certain distinct advantage. In addition, established cell lines such as MDCK cells serve as excellent models in which the cellular effects of hormones can be studied.

The first study undertaken was in MDCK cells which helped to establish the relationship between PAF and $[Ca^{++}]i$ in a stable and homogenous cell line. The results from this study prompted me to examine a similar relationship in human nasal epithelial cells.

In our first study (Chapter 2) we found that the $[Ca^{++}]i$ in MDCK epithelial cells responded in a dose dependent manner to PAF stimulation. The response was transient and could be inhibited by the PAF receptor blocker WEB 2086 and by the L-type Ca^{++} channel blocker nifedipine. In addition, the increase in $[Ca^{++}]i$ was reduced by removal of extracellular calcium. These results support the hypothesis that a PAF specific receptor exists on the surface of epithelial cells and that stimulation of the receptor leads to an increase in the $[Ca^{++}]i$. One very important piece of information that resulted from this study was the involvement of dihydropyridine-sensitive Ca^{++} channels in the PAF response. Currently, it is unknown how Ca^{++} transport occurs in non-excitable cells, such as epithelial cells. There have been reports of second-messenger activation of L-type voltage-gated Ca^{++} channel in response to hormone stimulation in adrenal cortical cells and in insulin-secreting pancreatic acinar cells (Heschler *et al.*, 1988; Velasco *et al.*, 1988). Our data suggest the involvement of a dihydropyridine-sensitive Ca^{++} channel in the PAF response which may provide an important clue to the understanding of Ca^{++} transport in epithelial cells.

In this first study we also found that the apparent EC50 value of 5.8×10^{-6} M for the PAF receptor activation was different from the 1.7×10^{-8} M value for pig kidney epithelial cells (Ueda *et al.*, 1991). The difference between these two values may be indicative of

different receptor subtypes on these two cell lines. Further investigation will be necessary to more fully define the PAF receptor characteristics in different cell types. The first study was done in a dog kidney epithelial cell line; the results from this study lend insight into the characteristics of the PAF response in all epithelial cells. Differences in cell types yielded somewhat different results as mitigating circumstances such as receptor subtype, receptor population and species differences exert their influence.

Based on the findings of the first study we undertook a similar but more detailed examination of the PAF receptor mechanism in explant-cultured human nasal epithelial cells. The study was designed to further elucidate the intracellular pathways that were involved in transmitting and interpreting the signal from the PAF receptor.

As found in the MDCK cells, the $[Ca^{++}]i$ in the human nasal epithelial cells responded in a dose dependent manner to PAF stimulation. This response could be inhibited by a PAF receptor blocker and could be reduced by decreasing the amount of free extracellular Ca^{++}. In addition, this study demonstrated that the signal from the PAF receptor was transmitted intracellularly by an N-ethylmaleimide-sensitive G-protein and that protein kinase C and cyclooxygenase products play modulating roles in the PAF response. While the increase in $[Ca^{++}]i$ was transient in MDCK cells, this study revealed that PAF stimulation leads to a sustained increase in $[Ca^{++}]i$ in human nasal epithelial cells. As sustained elevated $[Ca^{++}]i$ is toxic to cells and epithelial denudation is one aspect of asthma, it could be speculated that the inflammatory response in asthma leads to

epithelial sloughing due to toxic levels of $[Ca^{++}]i$ brought about by stimulation with PAF and other inflammatory mediators.

This project may have played a pivotal role in defining more precisely the mechanism of action of PAF in epithelial cells. By understanding the cellular mechanisms of action of PAF on epithelial cells, we may gain insight into the physiological results of cellular activation. And, by understanding the physiological activities that are enhanced by PAF stimulation we may be able to better understand the roles that PAF, $[Ca^{++}]i$ and epithelial cells play in physiological and pathological conditions.

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